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CYTOGENETIC BIODIVERSITY IN THE *Simulium johannseni* GROUP (DIPTERA: SIMULIIDAE)

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CYTOGENETIC BIODIVERSITY IN THE *Simulium johannseni* GROUP (DIPTERA: SIMULIIDAE)

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ABSTRACT

Cryptic biodiversity, the presence of suborganismal variation in isomorphic populations of organisms, can have implications for management and conservation. Ecological variation in habitat and host species of populations of the black fly *Simulium johannseni* suggested the potential for cryptic biodiversity. Polytene chromosomes of larvae belonging to the *S. johannseni* group of black flies were characterized to discover fixed and polymorphic rearrangements. Larvae of *S. johannseni* from Wisconsin, South Carolina, and Alabama and of the closely related *S. parmatum* from South Carolina and Florida were analyzed. *Simulium johannseni* can be divided into two cytoforms corresponding to geographic location. Cytoform A, found in Wisconsin, was selected as the chromosomal standard reference; it has 15 polymorphic inversions. Cytoform B, found in South Carolina and Alabama, has three fixed inversions and three polymorphic inversions. Cytoform B shares one polymorphic inversion with cytoform A; one of the fixed inversions in cytoform B is found as a polymorphism at low frequency in cytoform A. Further study is needed to determine if these two cytoforms represent separate species or extremes in clinal chromosome variation. *Simulium parmatum* has three fixed inversions and one polymorphic inversion, which is possibly sex-linked. *Simulium parmatum* shares two fixed inversions with *S. johannseni* cytoform B and has one unique fixed inversion. The polymorphic inversion in *S. parmatum* is shared with both cytoforms of *S. johannseni*, but in *S. parmatum* it was found only in the heterozygous state in males, suggesting it is Y-linked in *S. parmatum*. Evolutionary relationships were inferred based on shared inversions. The shared inversions indicate that *S. johannseni* cytoform B and *S.*

parmatum are more closely related to each other than to *S. johannseni* cytoform A. Further study of additional populations in the *S. johannseni* group and an outgroup are needed to test the relationships presented in the current analysis.

DEDICATION

I dedicate this thesis to my parents and sister for their love and support, and to Halli and Sarah, who provided sanity and understanding. Without you all, this would not exist.

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INTRODUCTION

The identification of species, already a contentious issue due to disputes over whether species should be defined based on reproductive isolation, ecological niche, phylogenetic lineage, or in terms of a discrete gene pool (Wheeler and Meier 2000, Claridge 2009, Hart 2011), is further complicated by the existence of cryptic (or sibling) species. Cryptic species are closely related and similar or indistinguishable anatomically, yet remain reproductively isolated (Adler et al. 2004). In organisms with polytene chromosomes, the presence of cryptic species can be inferred in sympatric populations from a lack of heterozygotes for distinct cytoforms, indicating reproductive isolation (Rothfels 1956). Cytoforms are populations of organisms with distinct chromosomal features. They might represent cytotypes (the cytological equivalent of morphological variants that are part of one interbreeding species) or cytospecies (species distinguished on the basis of distinct chromosomal banding patterns, with no evidence of interbreeding); the term cytoform is useful in cases in which evidence is not sufficient to determine whether separate banding patterns represent cytotypes or cytospecies (e.g., Pramual and Wongpakam 2011). The discovery and identification of cryptic species can have implications for conservation and management of biodiversity (Brown et al. 2007, Elmer et al. 2007).

Black flies (Diptera: Simuliidae) are biting flies; in most species, females feed on blood (Adler et al. 2004). Many species feed preferentially on birds or mammals, though a few species are not host-specific (Adler et al. 2004, Malmqvist et al. 2004). Black flies can serve as significant pests and harbor parasites of their preferred hosts (Adler 2005).

Black flies are pests of the highly endangered whooping crane, *Grus americana*, and might have negative impacts on the nesting success of these birds at the Necedah National Wildlife Refuge (NWR) in Wisconsin (Urbanek et al. 2010, King and Adler 2012). In great tits, *Parus major*, nest abandonment has been shown to be higher when nests are infested with blood-feeding hen fleas, *Ceratophyllus gallinae* (Richner 1998). Thus, the major pest species of the cranes, the black flies *Simulium annulus* and *S. johannseni* (Urbanek et al. 2010, King and Adler 2012), might influence the long-term success of the introduced whooping crane population. Although *S. annulus* did not historically overlap significantly with the cranes' nesting grounds, *S. johannseni* did (Adler et al. 2004), and thus historical populations of cranes might have evolved behaviors for dealing with high numbers of *S. johannseni*, for example, initiating nesting after the peak of *S. johannseni* adult emergence. *Simulium johannseni* adults near Necedah NWR in Wisconsin have their peak emergence in May, and *S. annulus* adults around the refuge emerge slightly earlier, peaking in abundance in late April (Adler 2009, Adler et al. 2010a). Whooping cranes at Necedah NWR begin their nesting around early May, which is a time of year more appropriate for latitudes in Maryland, where the initially established adult cranes were raised (Stehn 2001). The cranes have shown some adjustment to the latitude of the breeding ground in Wisconsin, initiating nesting efforts later in the year compared to prior breeding seasons (Adler, pers. comm.). *S. johannseni* might have a greater likelihood of influencing future crane nesting attempts than does *S. annulus*; although *S. annulus* might affect nesting currently (Urbanek et al. 2010, King and Adler 2012), *S. johannseni* is most abundant during the cranes' historical breeding

season for the latitude of Necedah NWR and thus might influence the cranes' nesting success as the cranes continue to adjust to the latitude of the breeding ground (Adler, pers. comm.).

The three described species in the *S. johannseni* species complex are *S. rothfelsi*, *S. parmatum*, and *S. johannseni*, including specimens formerly identified as *S. duplex* (Adler et al. 2004). The only confirmed record of *S. rothfelsi* is from Nova Scotia, Canada, although adults probably belonging to the species have been seen in the northeastern United States (Adler et al. 2004). *Simulium johannseni* is distributed in the central plains of the United States and Canada, and has populations along the southeastern coast of the Atlantic Ocean and along the Gulf of Mexico (Fig. 1; Adler et al. 2004). This disjunction between populations of the central plains and coastal plains might suggest the presence of cryptic species. Populations of *S. parmatum* have a distribution throughout the southeastern United States, overlapping with the coastal plain populations of *S. johannseni* (Fig. 2; Adler et al. 2004).

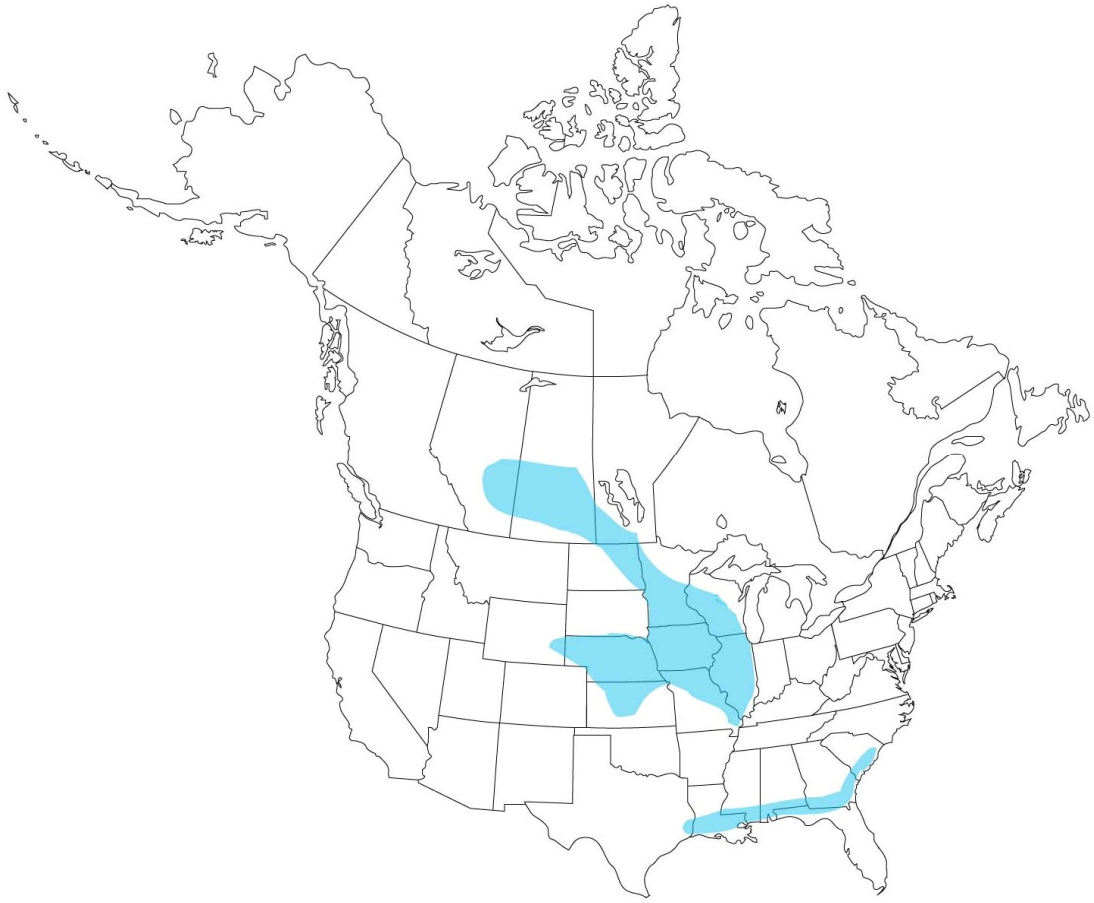

Fig. 1. Distribution of *Simulium johannseni* in North America. Blue areas cover approximate range based on reported collections of *S. johannseni* (Adler et al. 2004). The majority of recorded populations of *S. johannseni* were collected in the Great Plains of North America, although a few populations have been recorded in the southeastern coastal plain of the United States.

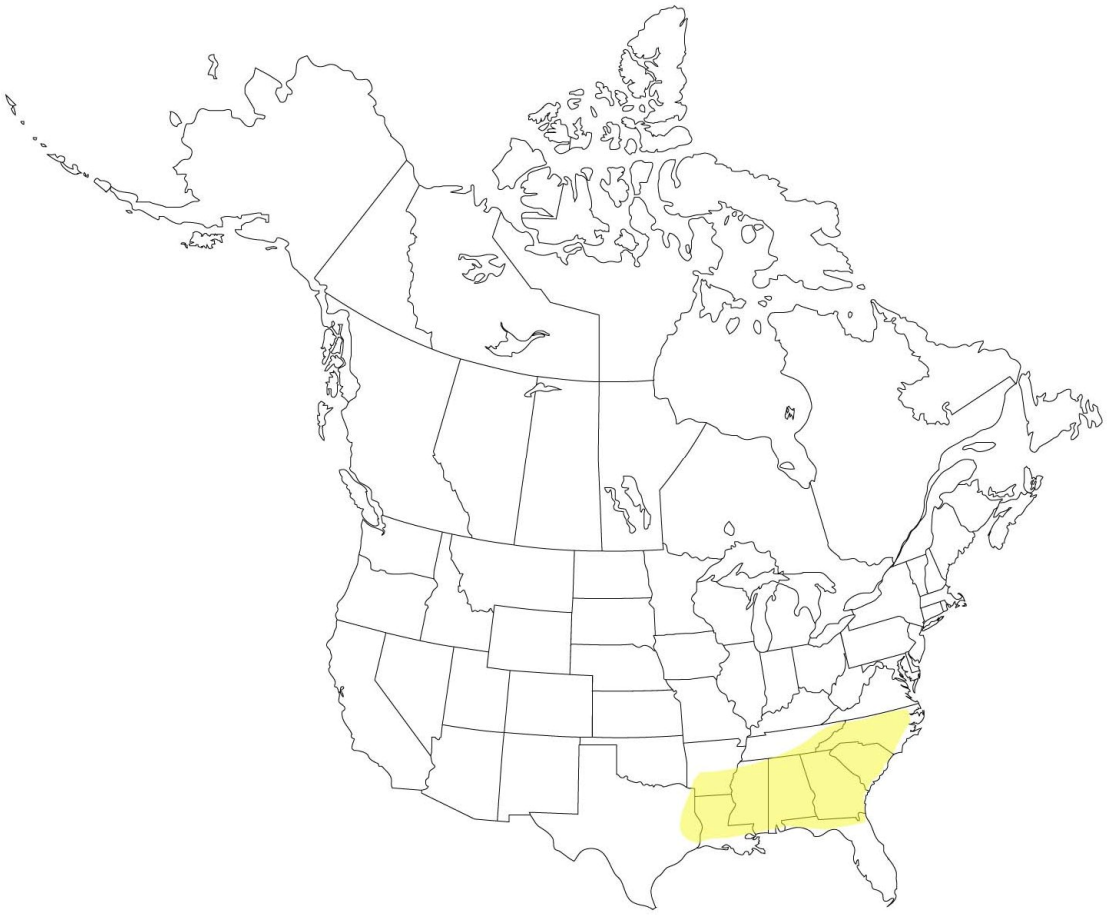

Fig. 2. Distribution of *Simulium parmatum* in North America. Yellow area encompasses approximate range based on reported collections of *S. parmatum* (Adler et al. 2004). *Simulium parmatum* has been recorded from locations throughout the southeastern United States.

In black flies (Simuliidae), cryptic species can be discovered and identified by differences in banding patterns of their giant polytene chromosomes (Adler et al. 2004). Populations of black flies are identified as having cryptic species by the presence of fixed inversion differences, differentiated sex chromosomes, and unique intraspecific polymorphic inversions, or at least two of these characteristics (Bedo 1977). Polytene chromosomes have been used to detect and identify cryptic species in tephritid fruit flies

(Diptera: Tephritidae) (Zacharopoulou et al. 2011) and mosquitos (Diptera: Culicidae) (Coluzzi et al. 2002). The identification of cryptic species of *S. johannseni* could be achieved by examining chromosomes of larvae and might have implications for management of black flies as pests of whooping cranes and other birds. The presence of cryptic species of *S. johannseni* around Necedah NWR could allow targeted management techniques specifically for those species that are pests of breeding whooping cranes.

Objectives and Hypotheses

No reports have been published regarding the presence of cryptic species in the Necedah population of *S. johannseni*. My research will result in chromosomal characterization of members of the *Simulium johannseni* group and identification of any cryptic biodiversity. The research will involve a focused examination of the Necedah population of *S. johannseni* and a subsequent examination of other populations of *S. johannseni* and *S. parmatum*. This work will enable me to infer evolutionary relationships based on chromosomal rearrangements. My work will help determine the potential for *S. johannseni* and local cryptic species to affect nesting of whooping cranes in Necedah National Wildlife Refuge. I will test the hypothesis that the Wisconsin population of *S. johannseni* near Necedah NWR contains undescribed, cryptic biodiversity (cytoforms or species), as suggested by the recent introduction of a new host, the whooping crane, and *S. johannseni*'s reported use of both mammal and bird hosts. I will also test the hypothesis that populations of black flies along the southern coast of South Carolina and Georgia and along the coast of the Gulf of Mexico, which are currently recognized as *S. johannseni*, are a different cytoform from *S. johannseni* populations in Wisconsin, as

suggested by the large geographic distance between these populations that could hinder gene flow, resulting in genetic divergence.

LITERATURE REVIEW

Polytene chromosomes

Polytene chromosomes are formed when DNA replicates but the duplicated strands do not separate; likewise, the cell itself does not undergo the full process of mitosis and remains a single entity instead of dividing into two daughter cells (Zhimulev and Koryakov 2009). Polytene chromosomes generally occur in tissues that experience rapid development and are involved in secretory activity (Zhimulev and Koryakov 2009). In black flies, these tissues typically are found during larval development and include the midgut epithelium, silk or salivary glands, and Malpighian tubules (Adler et al. 2004).

The replicated DNA strands (chromatids) coil without dividing, forming a single chromosome that pairs with its homologue, resulting in the classic type of polyteny, visible as large chromosomes with banding patterns characteristic of the species in which they are found (Zhimulev and Koryakov 2009). Banding patterns result from variation in chromatin concentration caused by variation in the amount of DNA coiling with histones and other proteins; dark areas indicate high concentrations of DNA and protein (Zhimulev and Koryakov 2009). These areas of tightly-packed DNA and protein might represent regions containing genes that are generally inactive in the tissue being examined. Early estimates of the amount of DNA in bands in *Drosophila* suggested that, on average, bands contain about 30 to 60 kb of DNA (Thomas 1941, Zhimulev and Koryakov 2009). More recent work has estimated bands to contain a wide range of quantities of DNA, from about 5 kb in very thin bands to over 200 kb in large, heavy bands (Zhimulev et al. 2012). The genome size of almost all black flies is unknown; the

only published genome size is a C-value of 0.19 pg, or 18.582 Mb, for *Prosimulium multidentatum* (Gregory 2011).

Biodiversity and Cryptic Species

Biodiversity refers to the number of different units of life in an area, often in terms of number of species, but biodiversity also represents the number of different genes or ecosystems in an area (Keesing et al. 2010). High biodiversity is considered a hallmark of healthy ecosystems, as the loss of biodiversity can have harmful results, including increased transmission of disease agents (Keesing et al. 2010).

Biodiversity can prove difficult to measure, in part because its definition is nebulous: if the unit of measure is number of species, the count may include only macroscopic organisms, only morphologically differentiated species, geographically distinct subspecies, or all species in an area, both microscopic and macroscopic, as well as all cryptic species. Many organisms are being revealed to have cryptic species, organisms that look the same but are reproductively isolated and therefore should be considered separate biological species (Elmer et al. 2007). Cryptic species may initially be recognized as subspecies, based on extensive population structure in a previously-defined species, which has been found in large vertebrates, including giraffes (Brown et al. 2007) and elephants (Rohland et al. 2010).

The presence of cryptic species presents conservation issues; cryptic species will have smaller numbers of individuals in their populations than were previously estimated for undivided morphospecies, raising the question of whether to classify each cryptic species separately on endangered species lists or keep the threat-level ranking for the

undivided morphospecies. Cryptic species typically have differing behavioral or biological characteristics (Rothfels 1981), which support separate threat rankings for individual threatened cryptic species. In cases where the cryptic species include pests, such as the blood-feeding of *S. johannseni* on newly-established migratory flocks of whooping cranes (Brooks et al. 2010), opportunities exist for targeted management. A pilot study on the effectiveness of *Bti* in controlling larval black flies was conducted near Necedah National Wildlife Refuge in April 2010, resulting in high levels of larval mortality for both *S. johannseni* and *S. annulus* (Adler et al. 2010a). Although the number of adult black flies collected at whooping crane nests in 2010 decreased compared to 2009, this decrease is probably not attributable to the limited *Bti* treatment, as collecting at nests in 2010 was limited compared to 2009 (Adler 2009, Adler et al. 2010a). The discovery of non-threatened cryptic species could have implications for the conservation of biodiversity; targeted management of possible cryptic black fly species for their effects on endangered whooping cranes would promote survival of the crane population while maintaining diversity of aquatic invertebrates in streams.

Identification of Cryptic Species

In many taxa, including Diptera (Ekrem et al. 2010), DNA "barcoding" can be used to identify cryptic species (Costa and Carvalho 2010). DNA barcoding involves sequencing and annotating the same section of the genome for each species of interest; these short sequences can subsequently be used to infer evolutionary history (Costa and Carvalho 2010). In black flies, banding patterns of the polytene chromosomes can be used to distinguish cryptic species (Rothfels 1981, Adler et al. 2010b). These same data

can be used to infer phylogenies, but these inferences must be approached cautiously, as gene (and presumably chromosomal) phylogenies do not necessarily follow species phylogenies (Avise 2004). Divergence in genes can occur without divergence in species (Avise 2004). Other problems can arise because of homoplasy or convergence (Sites and Marshall 2004), although the banding patterns of polytene chromosomes putatively are not subject to convergence (Rothfels 1981). Caution must also be exercised when looking for cryptic species in allopatric populations – sympatric populations featuring different fixed inversions may be inferred to be separate species, but allopatric populations with different inversions may simply represent a range of chromosomal variation throughout the species' geographic range (Rothfels 1989).

DNA markers can also be hybridized to polytene chromosomes, providing greater opportunities for tracking gene flow and determining the evolutionary history of related species, especially if in the Simuliidae, as in *Drosophila*, genes are typically syntenic, meaning homologous genes are found on the same chromosome arms across species, although the genes are often in a different order (Schaeffer et al. 2008). Giant polytene chromosomes, best viewed in the larval silk glands of the Simuliidae, can be used to determine population structure and evolutionary relationships, and to infer reproductive isolation based on banding patterns, which are conserved throughout the insect's life (Adler et al. 2004). In *Drosophila*, inversions are the major mechanism of genome change (Schaeffer et al. 2008); inversions are the most common chromosomal change in simuliids as well (Adler et al. 2004), suggesting that genes typically remain on the same chromosomal arms after inversions occur. Chromosomal inversions might promote

speciation by suppressing recombination within and around the inversion breakpoints (Faria and Navarro 2010; Jackson 2011). Inversions might in some cases prevent gene flow between populations long enough for additional genomic incompatibilities to arise and result in reproductive isolation through reinforcement (Noor et al. 2001; McGaugh and Noor 2012). In some *Drosophila* species, inversions associated with separate species are also associated with reproductive characteristics such as female preference and hybrid sterility (Noor et al. 2001). Sex-linked inversions in known *Drosophila* species have been shown to promote divergence by reducing hybrid fitness and preventing recombination (Khadem et al. 2011). Similarly, sex-linked inversions in black flies might promote divergence through reduced fitness of offspring of parents with different sex-linked inversions (Rothfels 1989). Thus, once inversions arise, the suppression of recombination and reduction of hybrid fitness can allow reproductive isolation to develop even in sympatric populations. The inversions that occur in different black fly lineages may have different evolutionary fates, including fixation, loss, maintenance as a polymorphism, or sex-linkage (Rothfels et al. 1978), which can help in distinguishing cryptic species. Inversions in black fly species are largely paracentric and do not involve the centromere (pericentric); sex-linked inversions are pericentric more than would be expected (Bedo 1977).

The *Simulium johannseni* group

Currently, 2,120 extant, formally named species of black flies are known (Adler and Crosskey 2012). Although the aquatic larvae are seen as beneficial in food webs, terrestrial adults are generally considered pests because females need blood meals to

mature their eggs (Adler et al. 2004). The three species currently considered part of the *Simulium johannseni* complex are *S. johannseni*, *S. parmatum*, and *S. rothfelsi*. *Simulium johannseni* was first described in 1912 (Forbes 1912). *Simulium duplex*, now included in *S. johannseni*, but formerly considered a subspecies or separate species, was described in 1958 (Shewell and Fredeen 1958). Populations of *S. johannseni* are found primarily in midwestern North America, from southern Alberta to southern Alabama, although individuals have also been reported from the southeastern coastal plain (Adler et al. 2004). This wide separation in habitat between the central plains of North America and the southeastern coastal plain might indicate the presence of cryptic species. Females of *S. johannseni* feed mostly on gallinaceous birds, including domestic poultry and ducks, and sometimes on mammals (Adler et al. 2004). Host switching is rare in other insect taxa. Bat flies (Diptera: Streblidae) exhibit high host specificity, specializing on one host species of bat in many cases (Dick 2007). Butterflies and moths are phytophagous as larvae and usually specialize on a single species of host plant or use a small number of closely related host plant species (Larsen et al. 2008, Bauer et al. 2012). The diversity of host species used by *S. johannseni* – populations in Minnesota are managed because they are a pest of humans (Adler, pers. comm.) – might be indicative of cryptic species, as freely switching between mammalian and avian hosts is not common among black flies. Previous work has shown high levels of host specificity among different species of black flies (Malmqvist et al. 2004). *Simulium parmatum* was described in 2004 (Adler et al. 2004), and occupies habitats in the piedmont and sandhills of the southeastern United States (Fig. 2), overlapping with coastal plain populations of *S. johannseni* (Fig. 1; Adler

et al. 2004). *Simulium rothfelsi* was described in 2003 (Adler, Brockhouse & Currie 2003) with only one confirmed record from Nova Scotia, although adults may have been collected in the northeastern United States (Adler et al. 2004).

CHROMOSOMAL CHARACTERIZATION

Materials and Methods

Collection Sites

Larvae were collected from eight total sites, five in Wisconsin and one each in Alabama, South Carolina, and Florida (Figs. 3, 4). Larvae of *S. johannseni* were collected from the Yellow River and Lemonweir River near Necedah National Wildlife Refuge in the spring of 2010 by P. Adler (sites 1-6), from Murder Creek in Alabama in early 2011 by J.W. McCreadie (site 8), and from the Black River in South Carolina in the spring of 2011 by P. Adler (site 7; Fig. 4, Table 1). Larvae of *S. parmatum* were collected from Graves Creek in Florida in the spring of 2010 by J.W. McCreadie (site 10) and from the Black River in South Carolina in the spring of 2011 by P. Adler (site 9; Fig. 4, Table 1). Geographic distances between sites ranged between 0 and 1582 km (Table 2). All larvae were collected into Carnoy's solution (3 parts 95-100% ethanol: 1 part glacial acetic acid) and kept in a freezer at approximately -20°C until prepared as slides. A total of 113 *S. johannseni* larvae were analyzed from five sites in Wisconsin, one site in South Carolina, and one site in Alabama (Table 1). Sites 2 and 3 were the same location, but larvae were collected on two different dates. A total of 16 larvae of *S. parmatum* were analyzed from one site in South Carolina and one site in Florida (Table 1). Voucher specimens of larvae collected from each site were deposited in the Clemson University Arthropod Collection as archival material.

Fig. 3. Collection sites for black fly larvae of the *Simulium johannseni* group. Collecting locations are marked in red, overlaid on the *S. johannseni* distribution (blue).

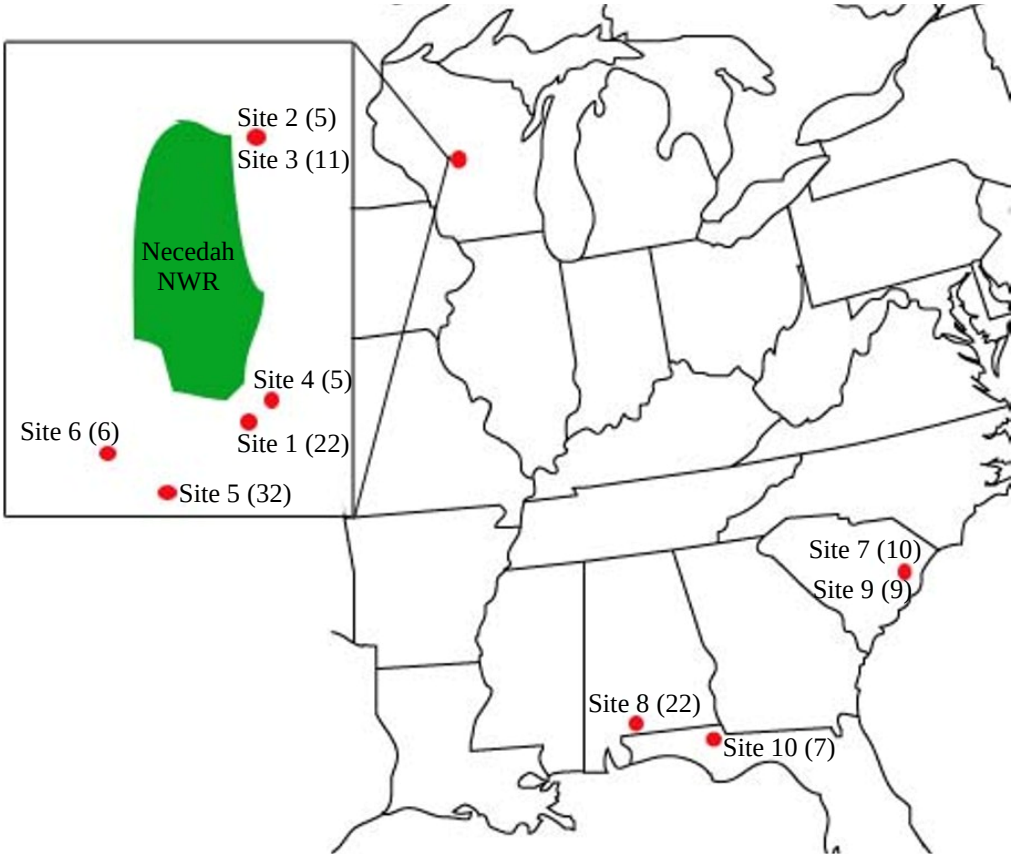

Fig. 4. Detailed map of collection sites for black fly larvae of the *Simulium johannseni* group. Red dots represent approximate locations of collecting sites; numbers in parentheses are total larvae analyzed for the site (n). Inset shows locations of sites in Wisconsin, near Necedah National Wildlife Refuge (green). Sites 2 and 3 in Wisconsin are the same location, collected on different dates and treated separately in analyses. Sites 7 and 9 in South Carolina are the same location treated separately in analyses depending on larval identity: *S. johannseni* larvae were grouped as site 7; *S. parmatum* larvae were grouped as site 9.

Table 1. Collection sites for black fly larvae of the *Simulium johannseni* group. N is the number of larvae completely scored for chromosomal banding pattern. Site 7 represents *S. johannseni* larvae collected in SC; site 9 represents *S. parmatum* larvae collected in SC.

Site no.	**State**	**County**	**River**	**Site**	**GPS Coordinates**	**Date collected**	**N**
1	WI	Juneau	Yellow	South Branch	44°00.614’ N 90°06.262’ W	31 Mar. 2010	22
2	WI	Juneau	Yellow	4th St.	44°12.816’ N 90°07.162’ W	30 Mar. 2010	5
3	WI	Juneau	Yellow	4th St.	44°12.816’ N 90°07.162’ W	5 Apr. 2010	11
4	WI	Juneau	Yellow	Rt. 21 & 80	44°01.547’ N 90°04.315’ W	31 Mar. 2010	5
5	WI	Juneau	Lemonweir	River Rd.	43°58.624’ N 90°14.874’ W	31 Mar. 2010	32
6	WI	Juneau	Lemonweir	24th-25th St.	43°59.896’ N 90°17.908’ W	31 Mar. 2010	6
7	SC	Williamsburg	Black	Rt. 377	33°35.176’ N 79°49.062’ W	18 Mar. 2011	10
9							9
8	AL	Escambia	Murder Creek	Mildred St.	31°06.036’ N 87°04.104’ W	31 Jan. 2011	22
10	FL	Gadsden	Graves Creek	Hwy 270A	30°37.581’ N 84°49.500’ W	1 Mar. 2010	7

Table 2. Straight-line distance between collection sites for larvae of the *Simulium johannseni* group to nearest km.

Site	1	2	3	4	5	6	7	8	9	10
1										
2	23									
3	23	0								
4	3	21	21							
5	12	28	28	15						
6	16	28	28	18	5					
7	1461	1479	1479	1461	1466	1471				
8	1462	1484	1484	1463	1460	1463	736			
9	1461	1479	1479	1461	1466	1471	0	736		
10	1561	1582	1582	1561	1561	1564	576	221	576	

Slide Preparation

To prepare chromosomes for analysis, larvae were dissected to expose the salivary glands and stain their chromosomes with Feulgen, using the technique of Rothfels and Dunbar (1953). The abdomen was removed and stained, with the head retained at -20°C in Carnoy's solution to be used as an additional method for identification if necessary. After staining, salivary glands were dissected, at which time the gonads were visible along the dorsal wall of the abdomen and the larvae could be sexed. Female gonads are long and thin, while male gonads are globular. Salivary glands and one gonad were placed in 50% acetic acid on microscope slides, where the glands were macerated before application of a cover slip to facilitate spreading of the nuclei. The entire chromosomal complement – three homologous pairs of chromosomes – was analyzed visually using a light microscope at 1250x magnification.

Standard Map Construction

Film photographs of selected chromosomes were taken under oil immersion (1250x) to be used in constructing a standard reference map for *S. johannseni* (Fig. 5). The film was scanned with a Nikon Coolscan V ED into a computer, and chromosome sections were pieced together using Adobe Photoshop CS2 (version 9.0.2) into a continuous map of each chromosome arm (Figs. 6-11). Chromosomes were numbered I, II, and III based on length, according to convention (Rothfels et al. 1978, Adler et al. 2004). Arms were defined as the length from the centromere to the end of the chromosome, with each chromosome divided into a short (S) and long (L) arm. Conventional cytological landmarks were identified (Rothfels et al. 1978, Adler et al.

2004). The standard map was arbitrarily chosen based on the most prevalent banding patterns found in larvae collected from Wisconsin. Specimens were analyzed against the standard reference map for rearrangements in banding patterns throughout the entire chromosomal complement. The standard was adjusted during the course of scoring individual slides if identified inversions were found to be at a high frequency in the Wisconsin populations. For example, inversion IIL-3 (Fig. 9) was initially scored as standard with the gray band distal (farther from the centromere) and inverted with the gray band proximal (closer to the centromere). After realizing the high frequency of that inversion as initially defined (0.84 at site 1, fixed in all southern individuals) it was redefined, with the standard pattern having the gray band proximal rather than distal (Fig. 9). Larvae scored as homozygous for the standard banding pattern, using the initial standard sequence, were redefined as homozygous for the inverted sequence, and larvae initially scored as homozygous inverted were redefined as homozygous for the standard sequence; heterozygous larvae remained heterozygous. This redefinition changed the frequency of the inversion (0.16 at site 1, absent from all southern individuals). Cytoforms were identified based on shared inversions; populations sharing or lacking the same suite of fixed inversions were grouped together as representatives of cytoforms.

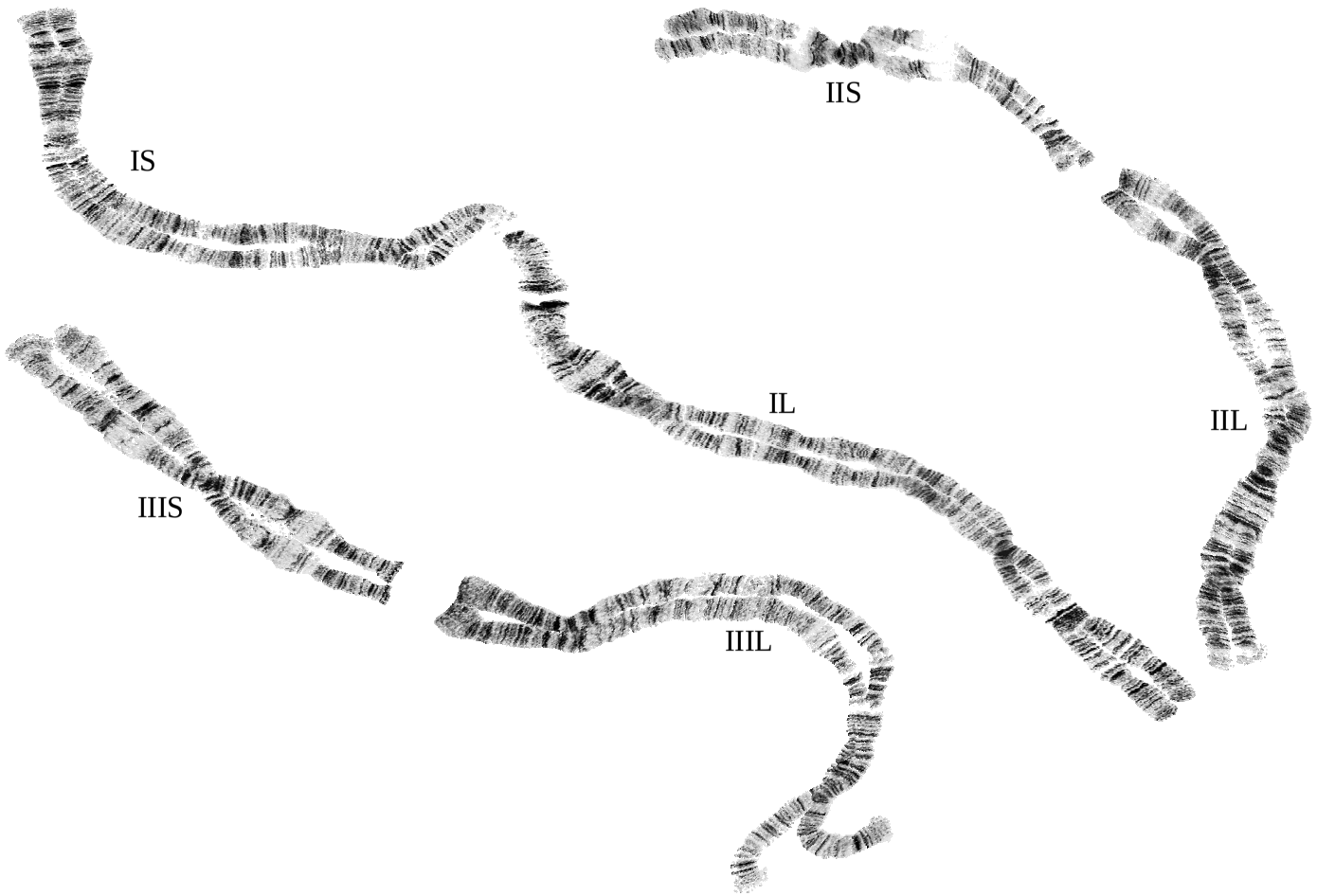

Fig. 5. Total polytene complement of *Simulium johannseni*, representing the standard banding sequence. Arms are identified with number (I, II, III) and letter (S = short, L = long). Figs. 7-12 provide detailed views of each arm delineating inversion breakpoints and conventional landmarks.

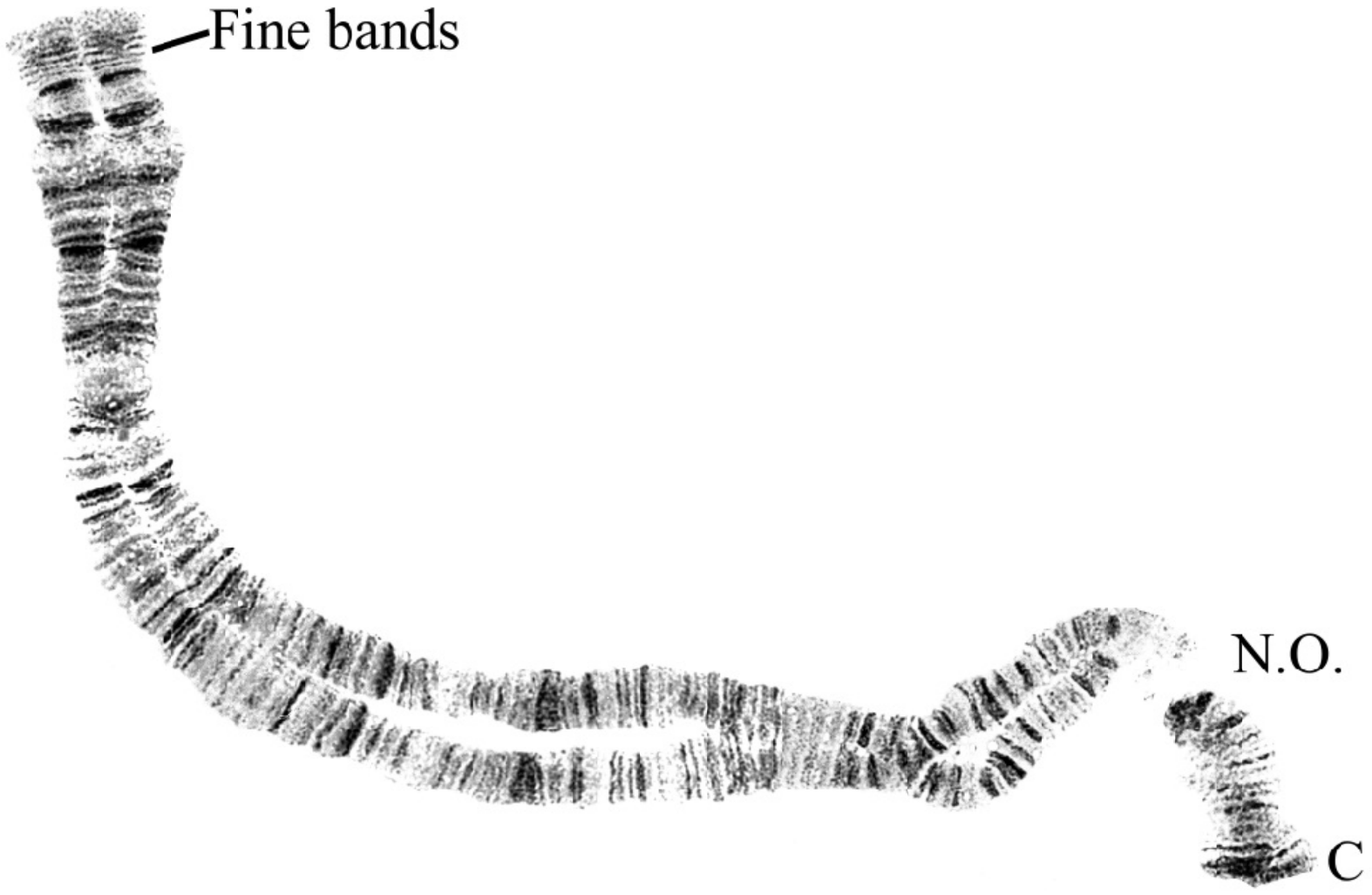

Fig. 6. Standard map of the short arm of chromosome I of *S. johannseni* (IS). The centromere (C) is at the lower right corner. Immediately distal to the centromere is the nucleolar organizer (N.O.).

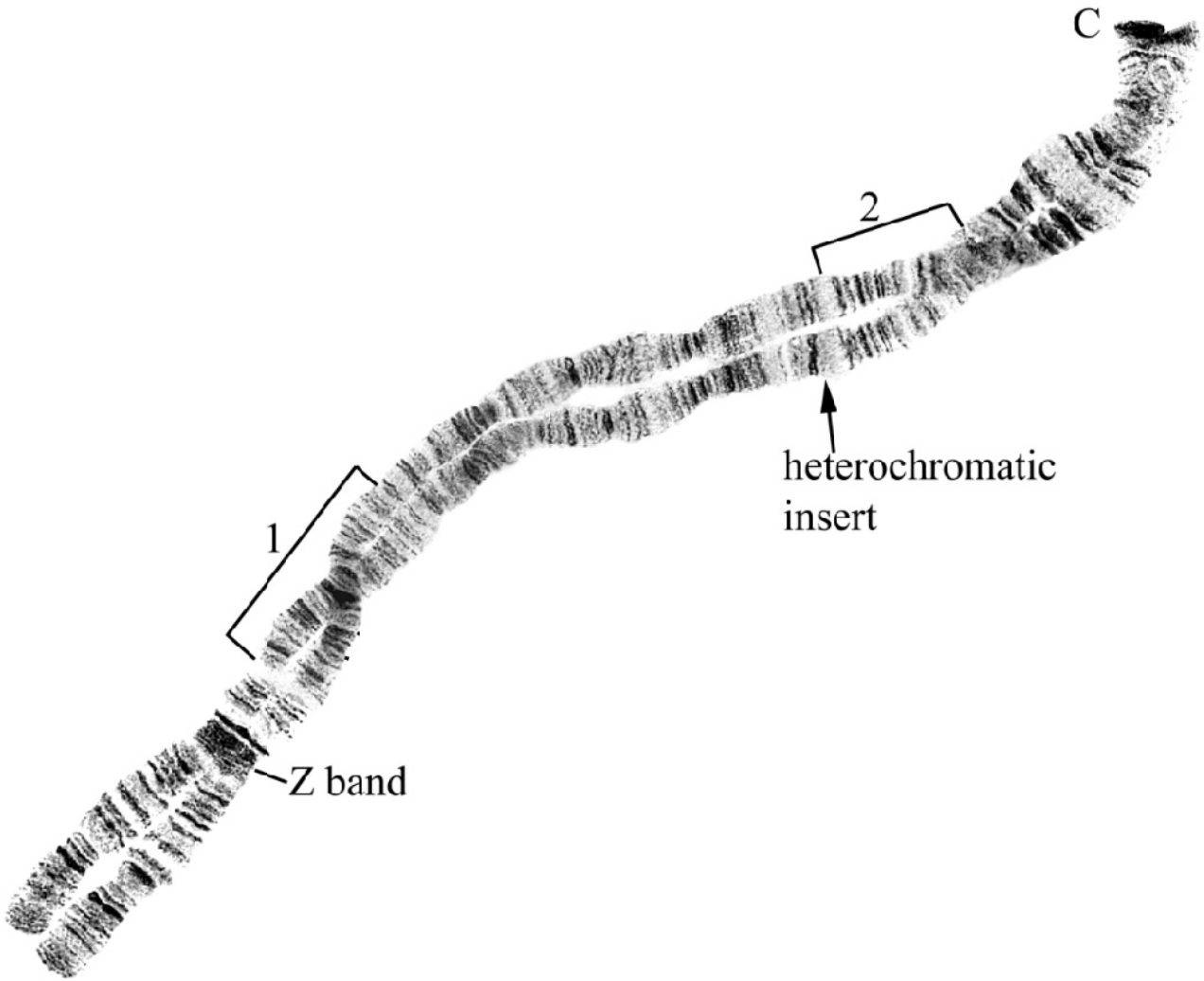

Fig. 7. Standard map of long arm of chromosome I of *S. johannseni* (IL). Two inversions were identified, as well as a heterochromatic band insert. The centromere (C) is at the top right corner of the photo. This arm features the Z band marker.

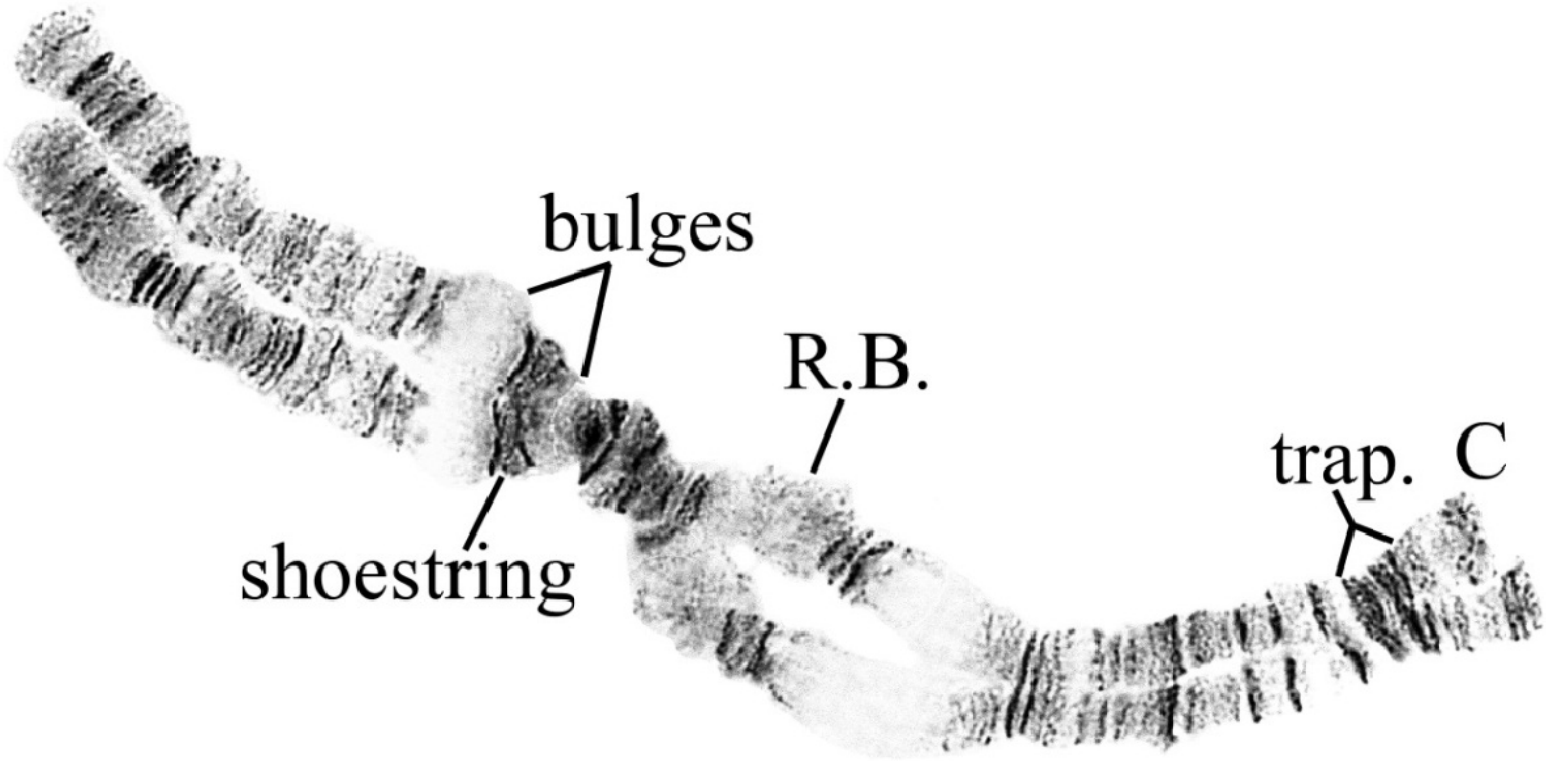

Fig. 8. Standard map of short arm of chromosome II of *S. johannseni* (IIS). The centromere (C) is at the right side of the photo. This arm features the bulges and shoestring, the Ring of Balbiani (R.B.), and the trapezoidal marker (trap). The secondary bulge proximal to the Ring of Balbiani is a structural polymorphism that was seen in some nuclei of some larvae.

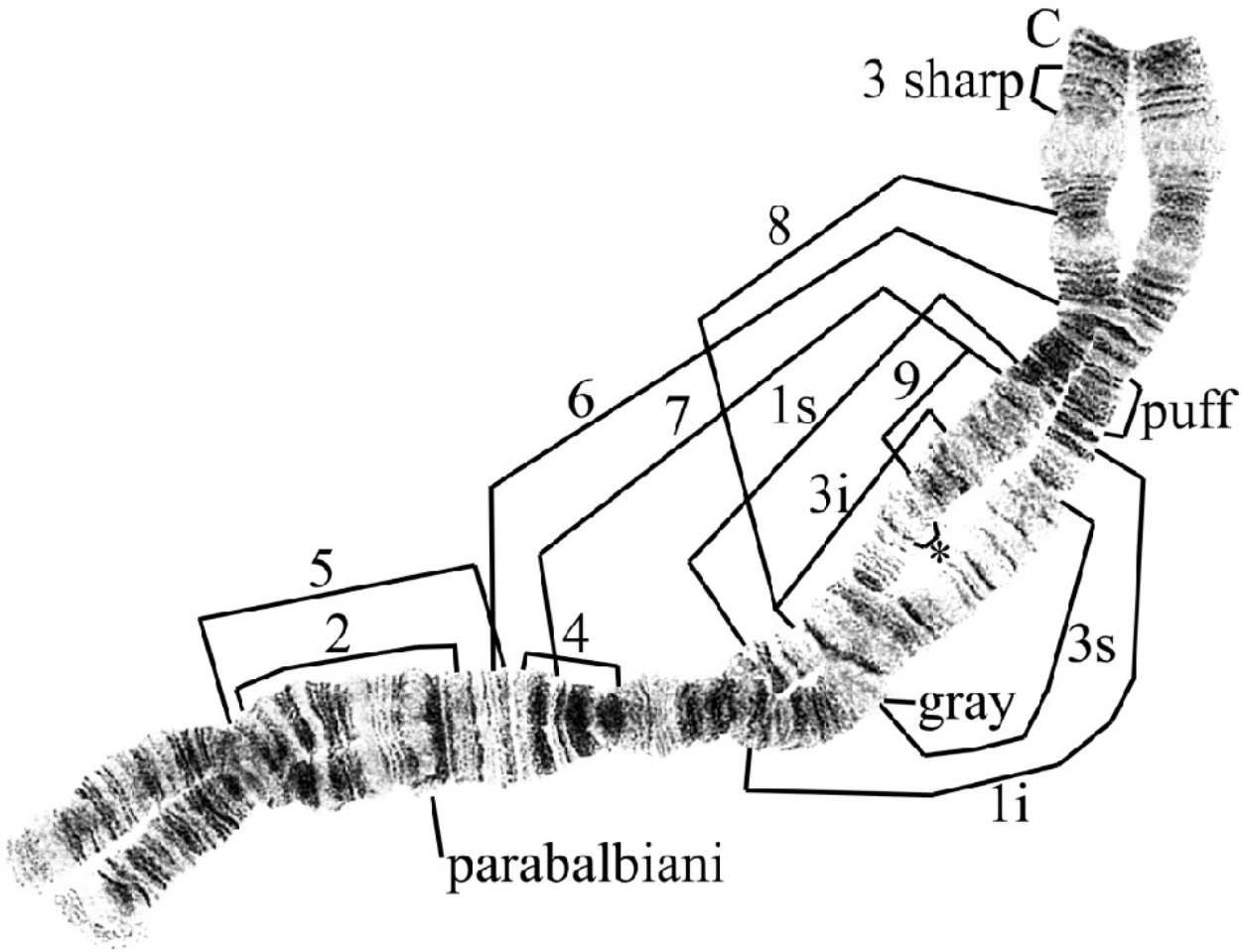

Fig. 9. Standard map of long arm of chromosome II of *S. johannseni* (IIL). The centromere (C) is at the top right corner. Nine inversions were found in this arm. This arm includes the 3-sharp bands, jagged (*), DNA puff, gray band, and parabalbiani. This map is heterozygous for inversions 1 and 3 (standard sequence of inversion 1 on top, standard sequence of inversion 3 on bottom).

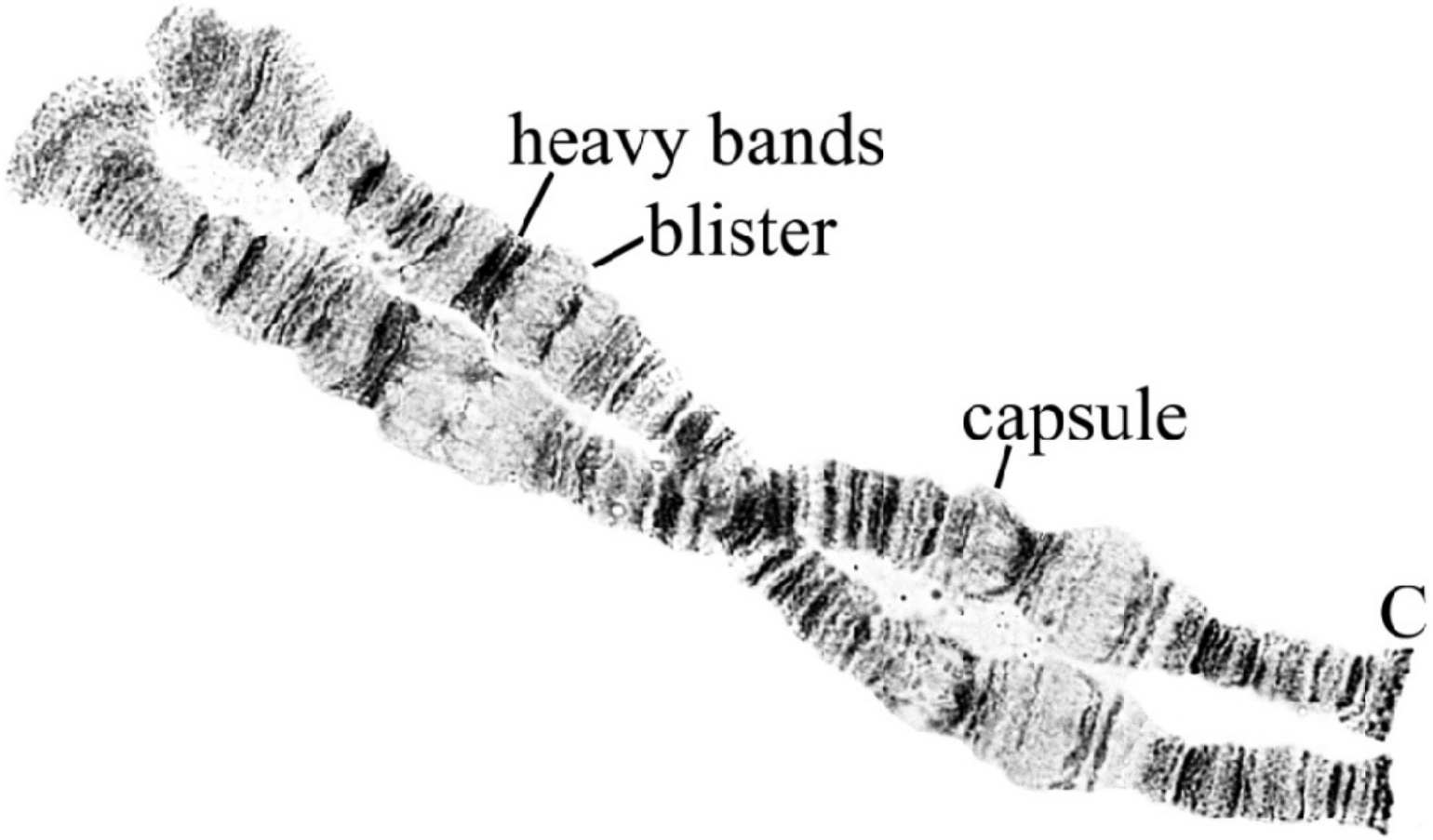

Fig. 10. Standard map of the short arm of chromosome III of *S. johannseni* (IIIS). The centromere (C) is at the right side. This arm includes the heavy bands, blister, and capsule.

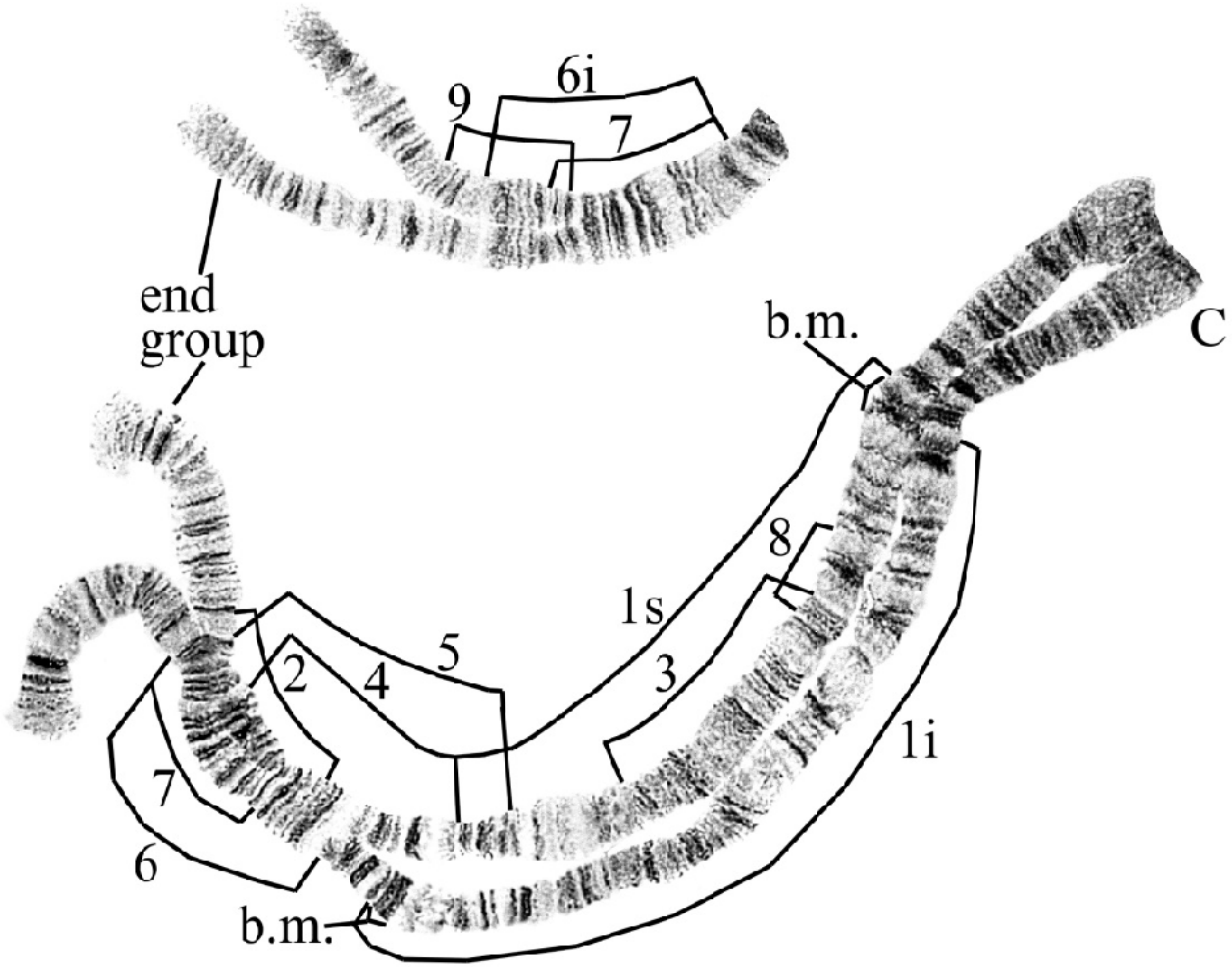

Fig. 11. Standard map of long arm of chromosome III of *S. johannseni* (IIIL). The centromere (C) is at the top right corner. Nine inversions were found in this arm; inversion IIIL-1 is heterozygous in this image. The inset, from *S. johannseni*cytoform B, shows inversion IIIL-9, which was found only in *S. parmatum* on top of inversion IIIL-6. This arm includes the basal marker (b.m.) and end group.

Statistical Analysis

Larvae were scored for their genotype class for identified inversions as either homozygous for the standard banding pattern (ss), heterozygous for the inversion of interest (si), or homozygous for the inversion of interest (ii). A Chi-square goodness of fit test was used to identify deviation from Hardy-Weinberg equilibrium for inversions in which a population had more than five individuals in each of two genotype classes. Expected genotype frequencies were calculated from allele frequencies, with the standard banding pattern represented as p and the inverted banding pattern represented as q, such that the expected frequency of standard homozygotes was p^2, the expected frequency of heterozygotes was $2pq$, and the expected frequency of inverted homozygotes was q^2. In sympatric populations, fixed inversions or significant deviation from Hardy-Weinberg equilibrium indicate the presence of cryptic species (Pramual and Wongpakam 2011). A G-test was used to evaluate possible sex-linked inversions. In many instances, sex-linked inversions initially arise in males and can be used to identify cryptic species (Shields and Kratochvil 2011).

Several tests were conducted to examine the degree of population genetic structure within the sampled populations. An inversion-by-site chi-square analysis was performed in SAS 9.2 to determine whether inversion frequency was associated with the site of origin. I used sequential Bonferroni corrections (Rice 1989) to account for multiple comparisons and considered inversions significantly associated with site if the p-value was less than 0.0025. For inversions found to be significantly associated with site, a subsequent pair-wise comparison of inversion frequency at that site was performed in

SAS 9.2 to reveal which sites had significant differences in inversion frequency. After accounting for multiple comparisons with the Bonferroni correction, sites were considered significantly different if the p-value was less than 0.001111. To corroborate population structure revealed in SAS, Arlequin 3.5 (Excoffier and Lischer 2010) was used to perform a genotype assignment test (Paetkau et al. 1995, 1997; Waser and Strobeck 1998) and to calculate pairwise F_{ST} values. In the assignment test, an individual's genotype is compared to the average genotype of each population and that individual is assigned to the most likely population of origin based on genotype frequencies. Arlequin calculates F_{ST} based on variance components summed over estimates at different hierarchical levels to estimate pairwise differences between populations (Excoffier and Lischer 2010). Previous studies have used F_{ST} values calculated from polytene chromosome inversion data to detect population structure (Pramual et al. 2009). A cytodendrogram was constructed using the most parsimonious groupings based on shared fixed inversions, with the chosen standard representing the hypothetical ancestral sequence. Additionally, Populations 1.2.32 (Langella 2011) was used to calculate Nei's standard genetic distance (Nei 1987) between each population and to construct a neighbor-joining tree (Saitou and Nei 1987), which was drawn using Phylip 3.69 (Felsenstein 2004).

Results

Allele Frequencies

Of 167 larvae stained and prepared for microscopic analysis, 129 (77.2%) were successfully scored for the entire chromosomal complement. Standard chromosomal maps were produced for each chromosome arm (Figs. 6-11). Twenty total inversions were found across all larvae, with nineteen inversions found across all *S. johannseni* larvae analyzed, fifteen in Wisconsin populations and six in southern populations (Table 3). Four inversions were present in the *S. parmatum* larvae, three of which were shared with *S. johannseni* (Table 4). Larvae collected in Wisconsin showed the highest amount of polymorphism, ranging from an average of 1.00 to 1.67 heterozygous inversions per larva per site (Figs. 12, 13). *Simulium johannseni* larvae collected in South Carolina and Alabama and *S. parmatum* larvae showed much less polymorphism than larvae collected in Wisconsin, with 0.22 to 0.59 heterozygous inversions per larva per site (Figs. 14-17). Larvae of both species collected from southern sites were fixed for inversions IIL-7 and IIIL-6. *Simulium johannseni* larvae from these sites were fixed for inversion IIL-6, whereas *S. parmatum* larvae were fixed for inversion IIIL-9. Thirteen of twenty inversions were found only in Wisconsin populations. Inversions IIL-6, IIL-7, IIIL-7, IIIL-8, and IIIL-9 were found only in southern populations (Figs. 14, 16). The consistent chromosomal differentiation between northern and southern populations of *S. johannseni* allows categorization into one of two cytoforms: cytoform A from Wisconsin, featuring polymorphic inversions and no fixed inversions, and cytoform B from South Carolina and Alabama, with fixed inversions IIL-6, IIL-7, and IIIL-6 (Figs. 18, 19).

Table 3. Summary of *S. johannseni* inversion data. Inversions IIL-3, IIL-6, and IIL-7 are fixed in the SC and AL populations.									
Site number		1	2	3	4	5	6	7	8
Number of males/females		14/8	3/2	5/6	5/0	14/18	4/2	6/4	8/14
Frequency of inverted constituent	IL-1	0.07	0.00	0.14	0.00	0.06	0.00	0.00	0.00
	IL-2	0.02	0.00	0.09	0.00	0.05	0.08	0.00	0.00
	IIL-1	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	IIL-2	0.16	0.00	0.14	0.00	0.13	0.08	0.00	0.00
	IIL-3	0.16	0.10	0.00	0.00	0.06	0.00	0.00	0.00
	IIL-4	0.02	0.00	0.05	0.00	0.06	0.00	0.00	0.00
	IIL-5	0.02	0.00	0.14	0.00	0.09	0.00	0.00	0.00
	IIL-6	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00
	IIL-7	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00
	IIL-8	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	IIL-9	0.02	0.00	0.05	0.00	0.02	0.00	0.00	0.00
	IIIL-1	0.39	0.20	0.32	0.30	0.31	0.50	0.00	0.00
	IIIL-2	0.02	0.20	0.14	0.00	0.13	0.00	0.00	0.00
	IIIL-3	0.07	0.10	0.23	0.10	0.19	0.25	0.15	0.14
	IIIL-4	0.05	0.00	0.05	0.10	0.00	0.00	0.00	0.00
	IIIL-5	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00
	IIIL-6	0.00	0.00	0.00	0.00	0.02	0.08	1.00	1.00
	IIIL-7	0.00	0.00	0.00	0.00	0.00	0.00	0.90	0.68
	IIIL-8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02

Table 4. Summary of *S. parmatum* inversion data. Inversions IIL-3, IIL-7, and IIIL-2 are fixed.			
Site number		7	9
Number of males/females		2/7	2/5
Frequency of inverted constituent	IIL-7	1.00	1.00
	IIIL-3	0.11	0.14
	IIIL-6	1.00	1.00
	IIIL-9	1.00	1.00

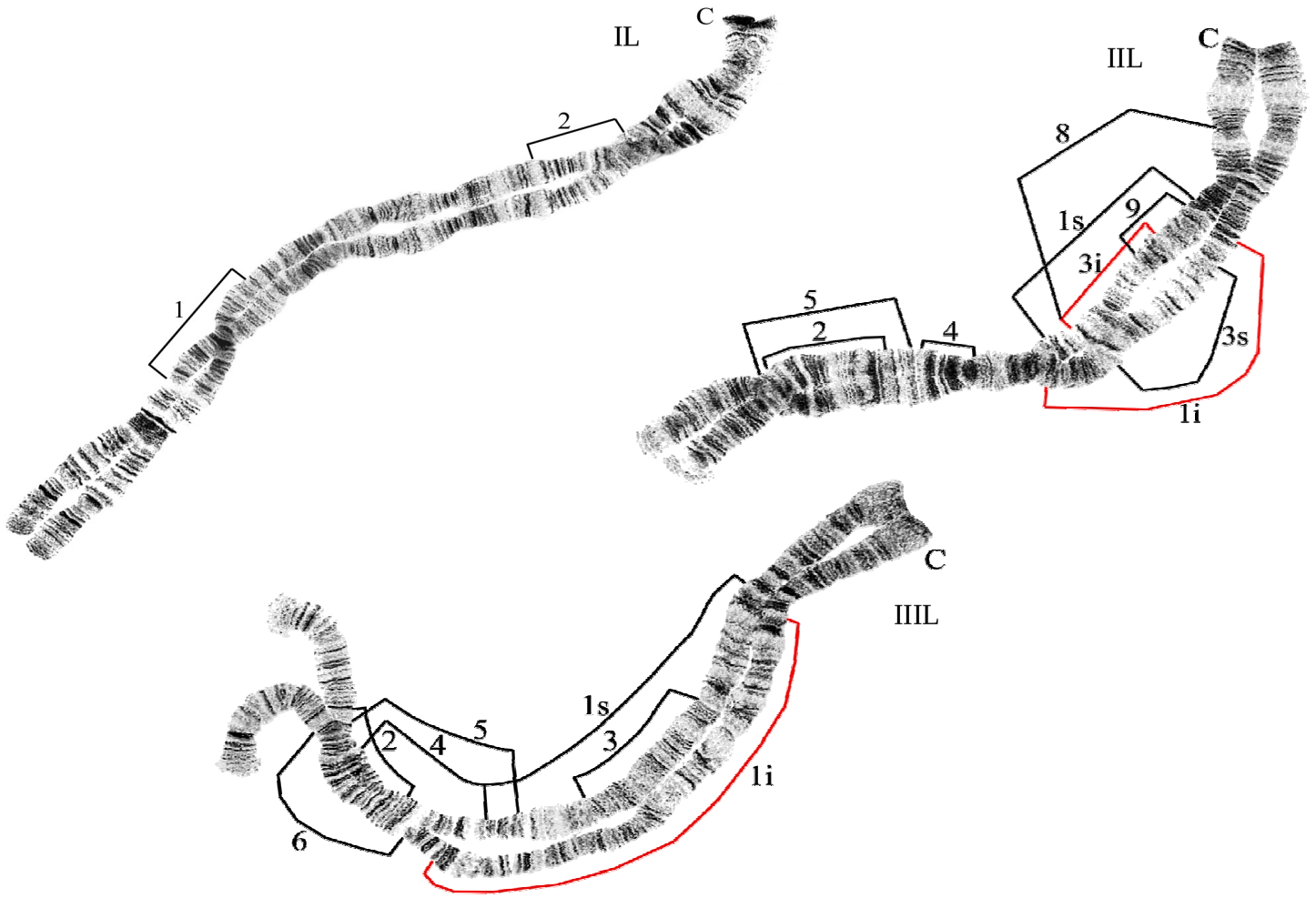

Fig. 12. Inversions found in Wisconsin populations of *S. johannseni*. Fifteen polymorphic inversions and no fixed inversions were found in Wisconsin. Inversions IIL-1 and IIL-3 are present heterozygously in this map; the inverted sequence is indicated with “i” and bracketed in red. Inversion IIIL-1 is present heterozygously in this map; the inverted sequence is marked with “i” and bracketed in red. “C” on each arm indicates the centromere.

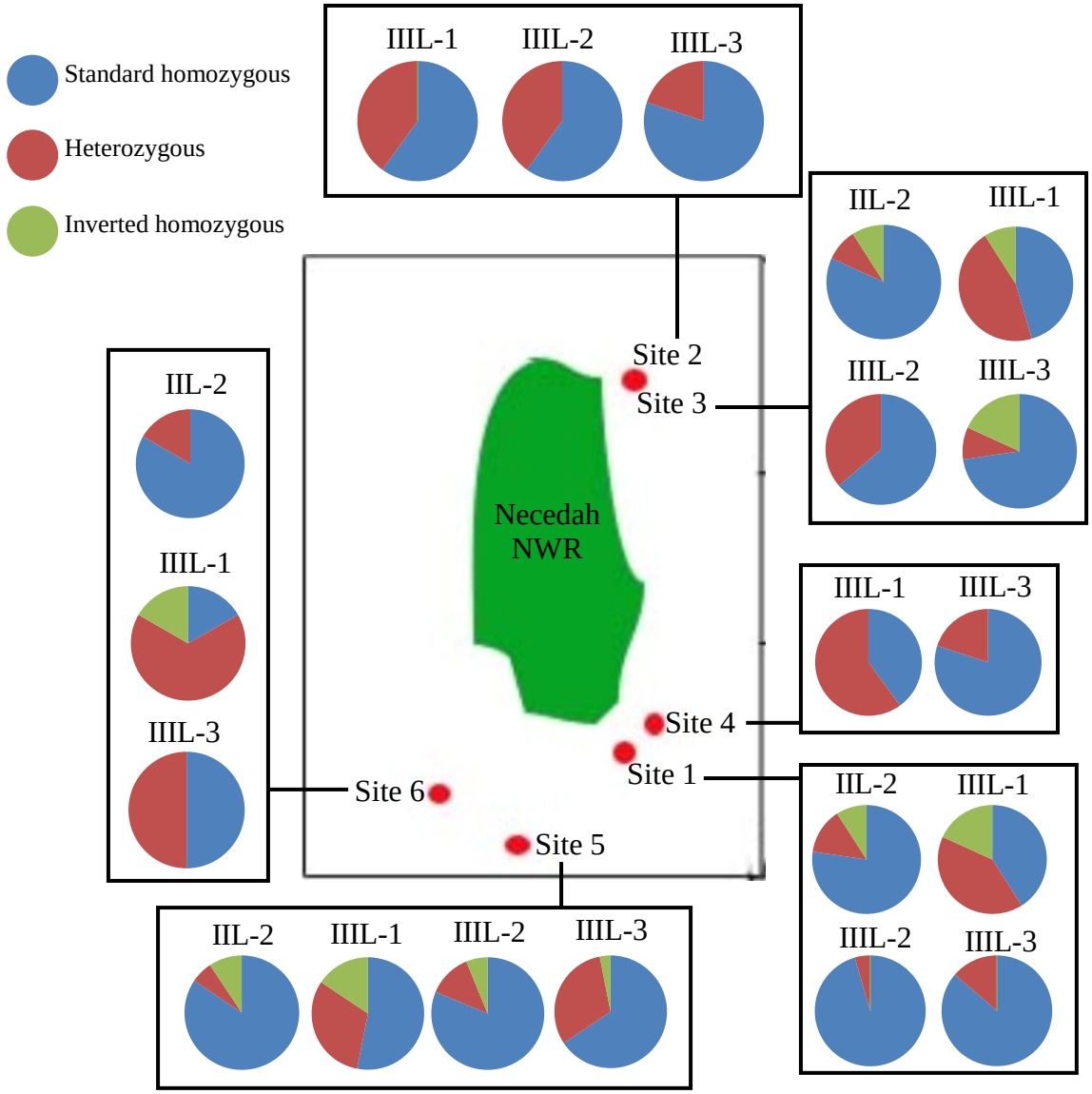

Fig. 13. Genotype frequencies of the four most common inversions found in *Simulium johannseni* in Wisconsin. Individuals homozygous for the standard sequence are blue, heterozygous individuals are red, and individuals homozygous for the inverted sequence are green in the pie charts of genotype frequency.

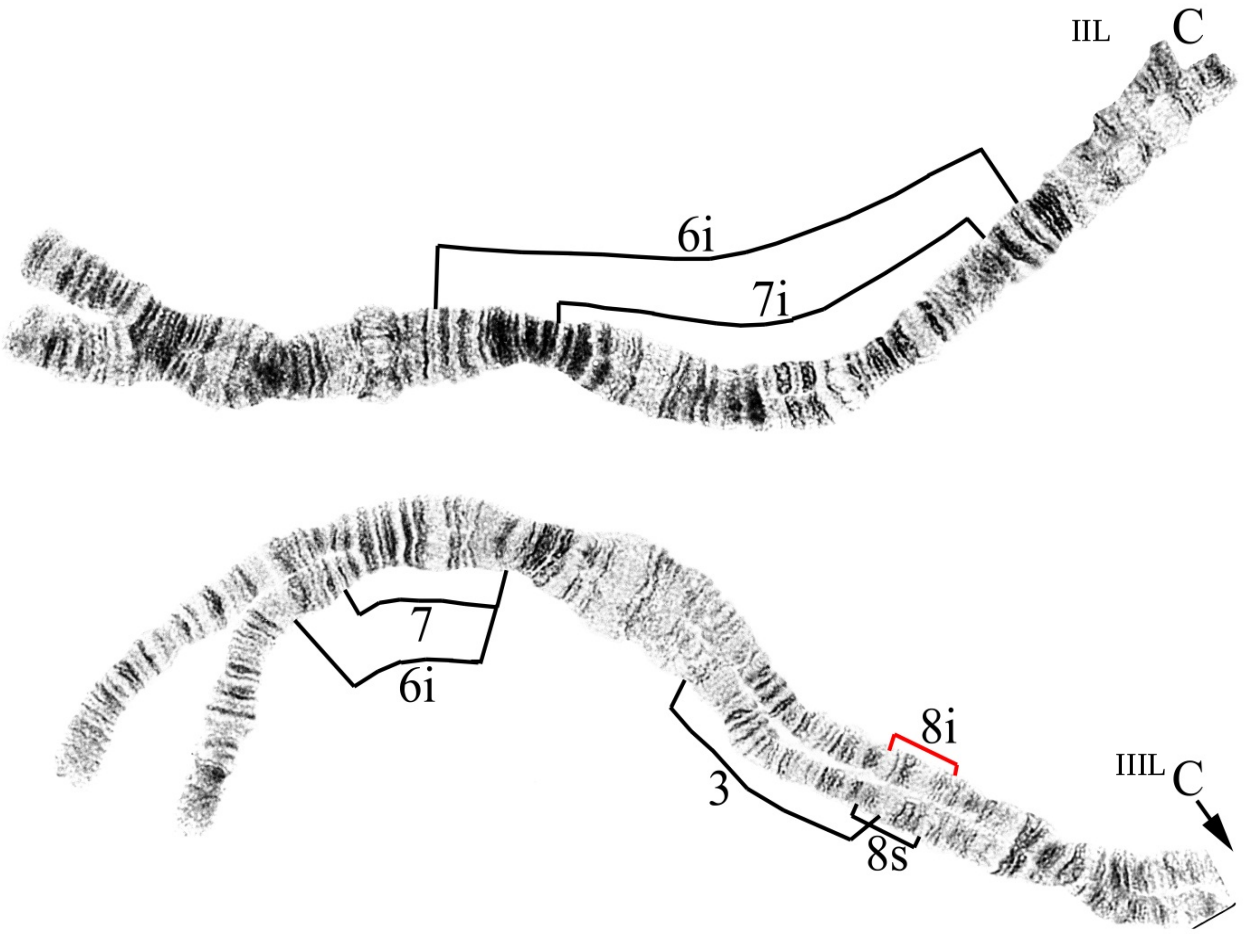

Fig. 14. Inversions found in southern populations of *S. johannseni*. Inversions IIL-6 and IIL-7 were fixed in the inverted pattern, as indicated with the "i." Inversion IIIL-6 was fixed in the inverted sequence, as indicated with the "i." Inversions IIIL-3, IIIL-7, and IIIL-8 were polymorphic. Inversion IIIL-8 is heterozygous in this map; the inverted arm is indicated with "i" and red bracket marking the breakpoints. "C" indicates the location of the centromere.

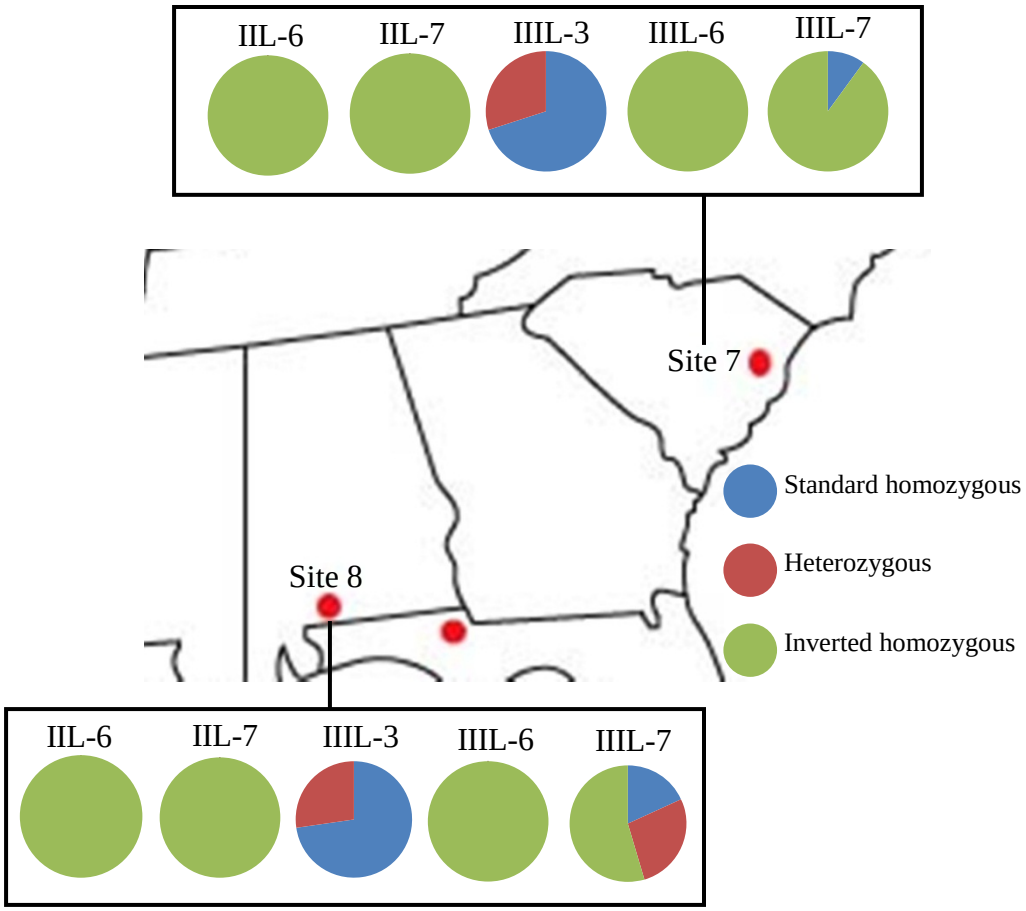

Fig. 15. Genotype frequencies of southern *S. johannseni*. Inversions IIL-6, IIL-7, and IIIL-6 were fixed for the inverted sequence, as shown by the completely green pie charts. Inversions IIIL-3 and IIIL-7 were the most common polymorphic inversions. Blue represents individuals homozygous for the standard sequence, red represents heterozygous individuals, and green represents individuals homozygous for the inverted sequence.

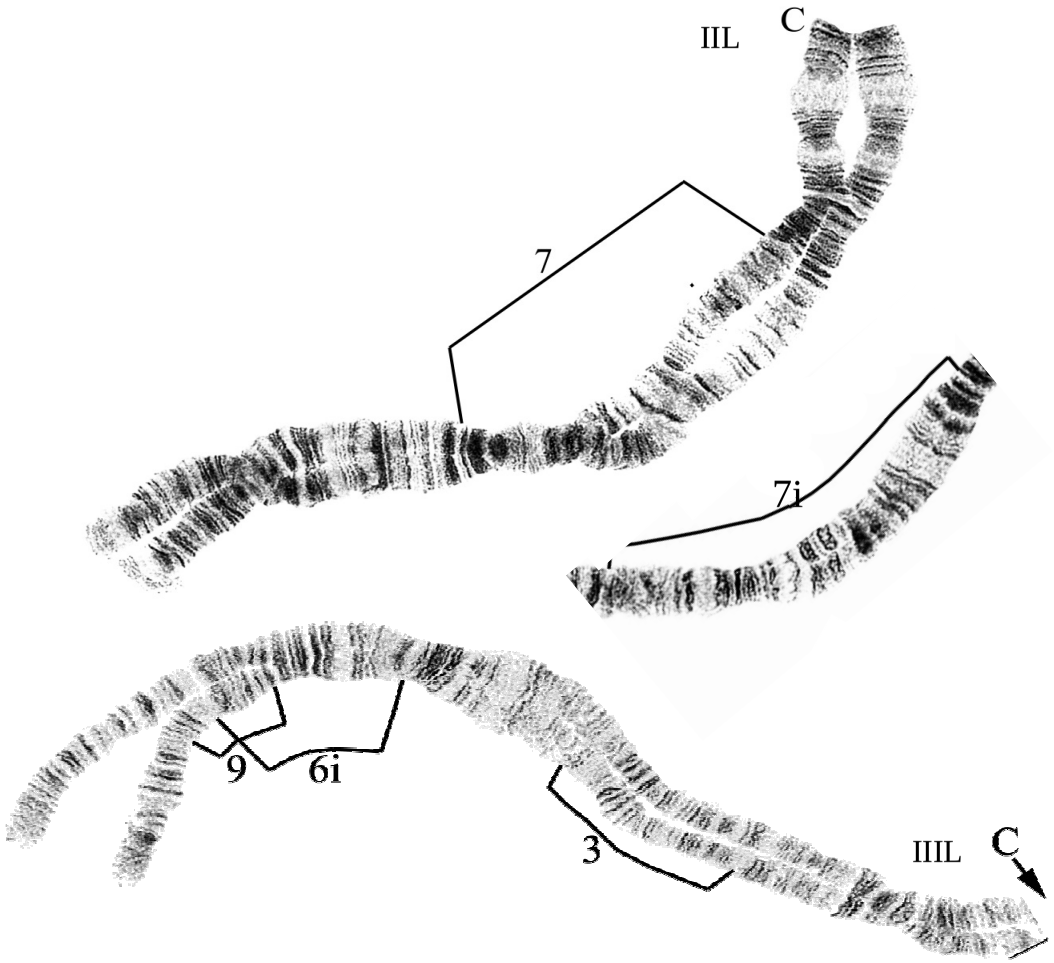

Fig. 16. Inversions found in *S. parmatum*, mapped on the standard sequence (top and middle) and on Cytoform B sequence (bottom). Inversion IIL-7 was fixed in the inverted sequence; the inset of "7i" shows the orientation of inversion 7 as it would be seen in *S. parmatum*. Inversions IIIL-6, shown here in the inverted form as "6i" and IIIL-9 were fixed; inversion IIIL-3 was polymorphic, but seen only in males heterozygously, suggesting possible sex-linkage. "C" indicates the centromere.

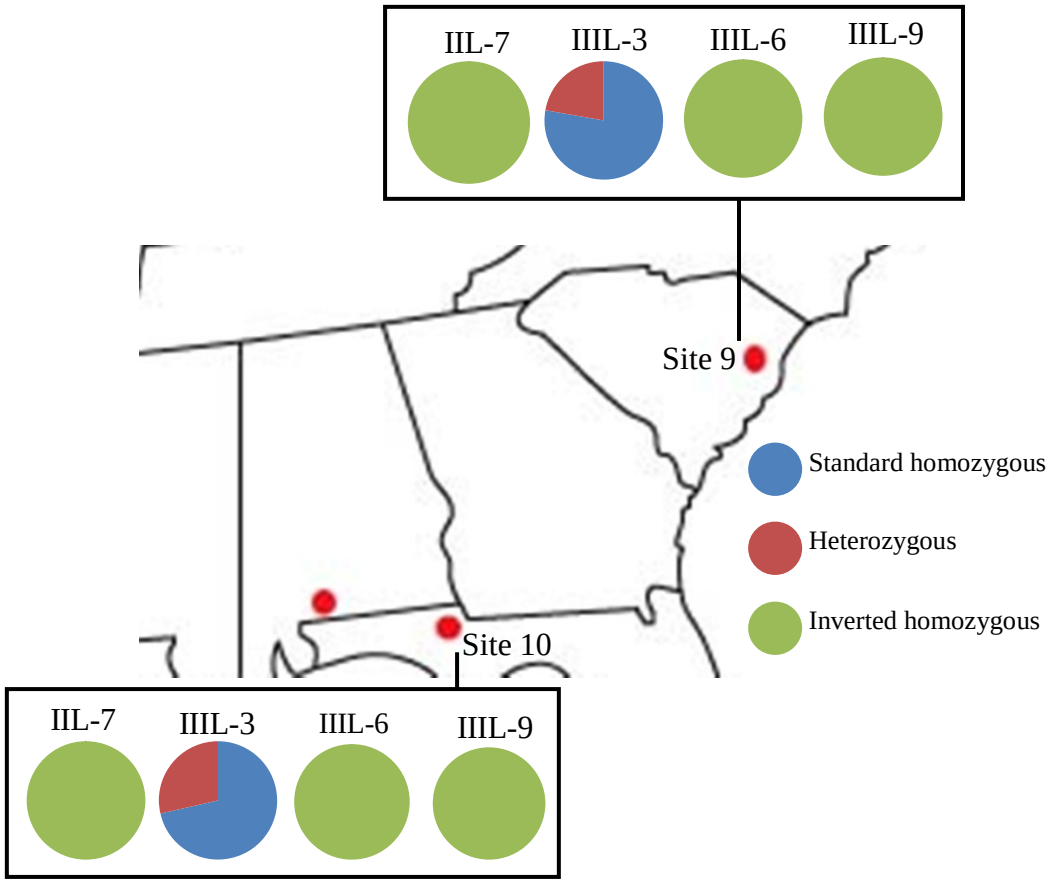

Fig. 17. Genotype frequencies of *S. parmatum*. All inversions found in *S. parmatum* are included. Inversions IIL-7, IIIL-6, and IIIL-9 were fixed. Inversion IIIL-3 was polymorphic, but found only heterozygously, and only in males, suggesting possible sex-linkage.

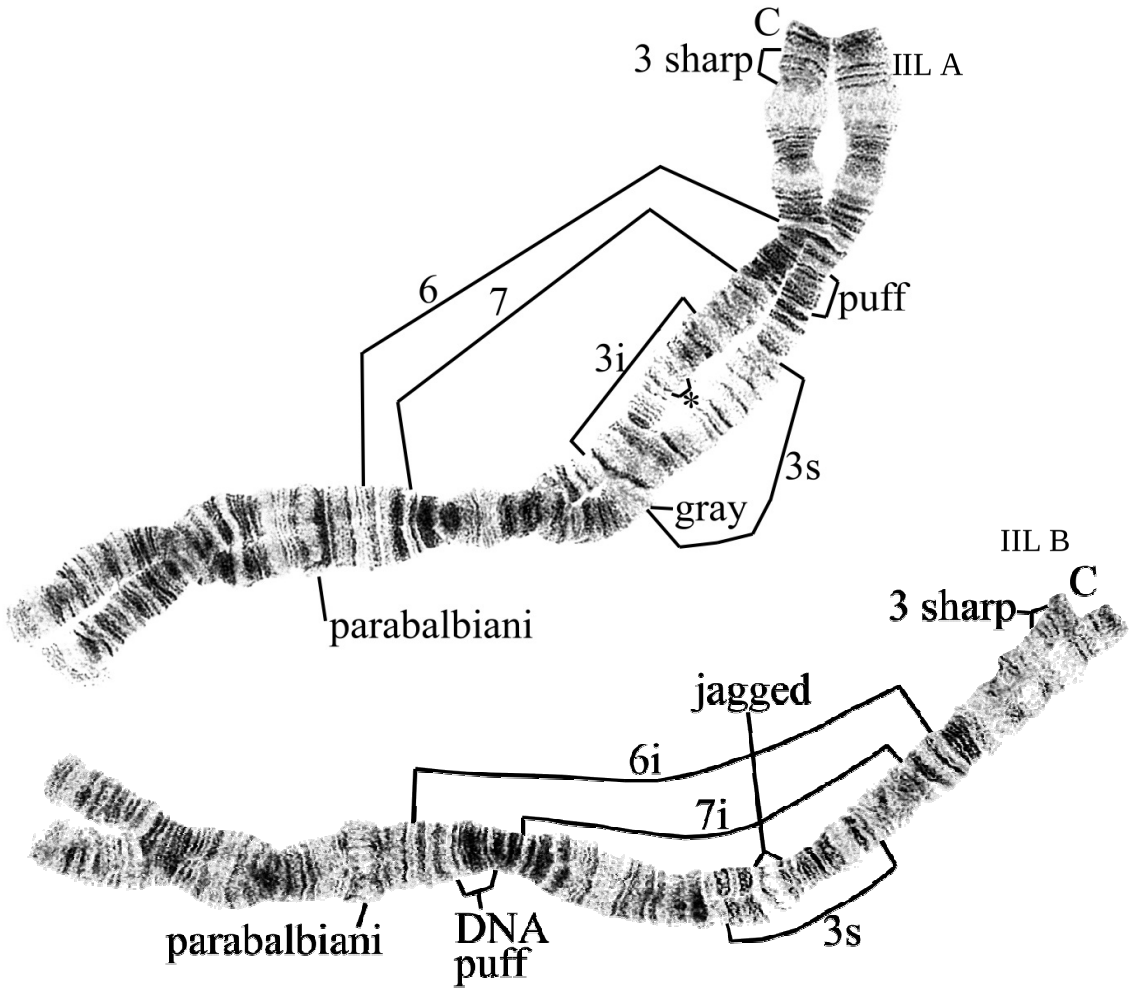

Fig. 18. Comparison of long arm of chromosome II between *S. johannseni* cytoform A (IIL A) and cytoform B (IIL B). Cytoform B, found in the southern populations, is fixed for inversions IIL-6 and IIL-7, causing the DNA puff to be located distally compared to its location in cytoform A. The * in IIA indicates the jagged landmark. “C” indicates the centromere.

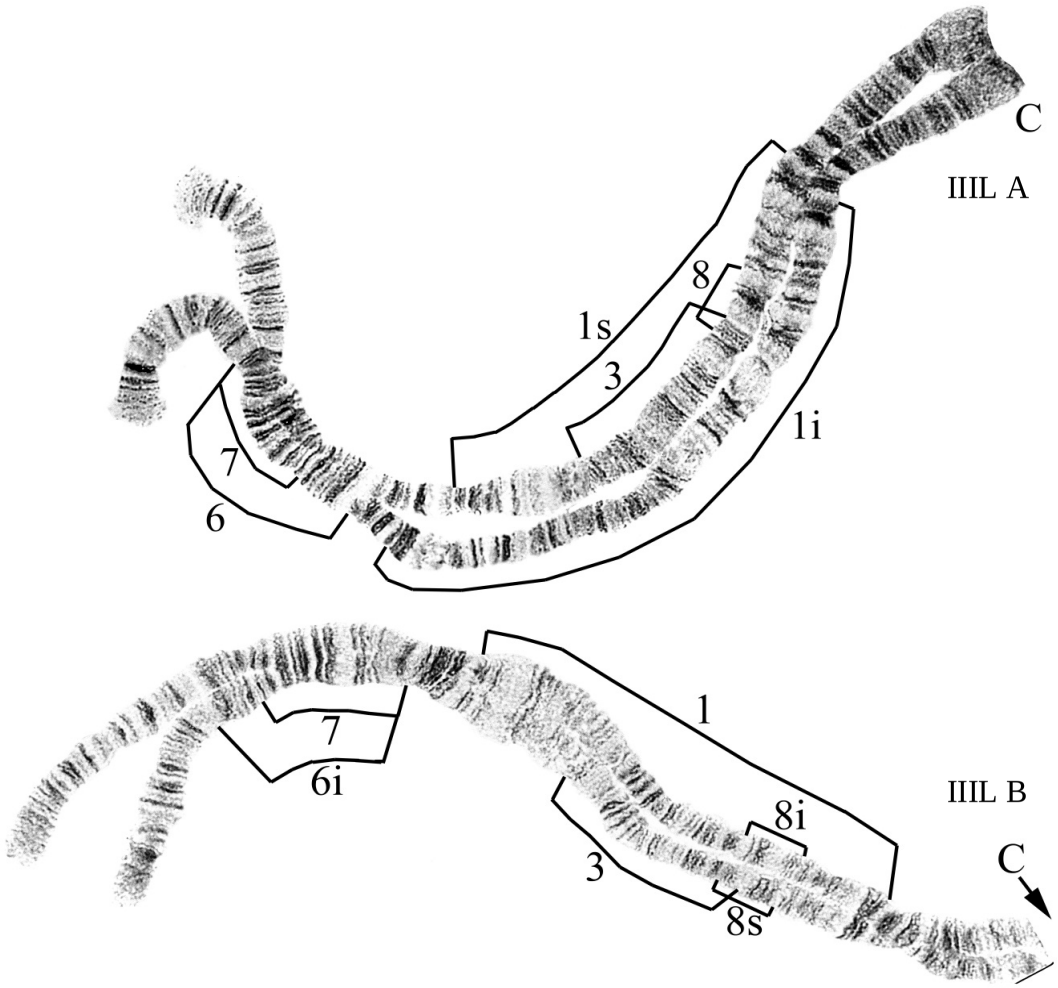

Fig. 19. Comparison of long arm of chromosome III between *S. johannseni* cytoform A (IIIL A) and cytoform B (IIIL B). Cytoform B, found in the southern populations, is fixed for inversion IIIL-6. Inversions IIIL-7 and IIIL-8 were polymorphic in cytoform B and not found in cytoform A. “C” indicates the centromere.

Conformation to Equilibrium Expectations

Of the inversions that had sample sizes sufficient for testing for deviation from Hardy-Weinberg equilibrium, none showed significant deviation from equilibrium expectations of p^2, $2pq$, q^2 genotype frequencies (Table 5). There were no indications of sex-linked inversions in any population of *S. johannseni* larvae. The site 1 population (South Branch, Yellow River, WI) had sufficient numbers of individuals to perform a G-test on inversion IIIL-1 and the site 5 population (River Road, Lemonweir River, WI) had sufficient numbers of individuals to perform a G test on inversions IIIL-1 and IIIL-3 (Table 6). None of the G-tests showed significant sex linkage. In the sixteen *S. parmatum* larvae analyzed, inversion IIIL-3 was found in the heterozygous condition only in the four males. The sample size was insufficient for conducting a G-test.

Table 5. Chi-square tests for Hardy-Weinberg equilibrium in populations of *S. johannseni* larvae. Chi-square values less than the critical value (with 1 degree of freedom) of 3.84 indicate a lack of significant deviation from Hardy-Weinberg equilibrium expectations.

Site	Inversion	Chi-square frequency table				χ^2 value	p-value
1	IIL-3		ss	si	ii	0.57	> 0.05
		Observed	1	5	16		
		Expected	0.53	5.62	14.85		
	IIIL-1		ss	si	ii	0.48	> 0.05
		Observed	9	9	4		
		Expected	7.91	9.96	3.13		
5	IIIL-1		ss	si	ii	2.38	> 0.05
		Observed	17	10	5		
		Expected	15.13	13.75	3.13		
	IIIL-3		ss	si	ii	0.02	> 0.05
		Observed	21	10	1		
		Expected	21.13	9.75	1.13		
8	IIIL-3		ss	si	ii	0.55	> 0.05
		Observed	16	6	0		
		Expected	16.41	5.18	0.41		
	IIIL-7		ss	si	ii	3.04	> 0.05
		Observed	4	6	12		
		Expected	2.23	9.55	10.23		

Table 6. G-test of independence for three inversions in larvae of *S. johannseni* from Wisconsin. Non-significant G values indicate independence of sex and inversion state.

Site	Inversion	G-test frequency table						G value	p-value
1	IIIL-1		ss		si		ii	5.70	> 0.05
			Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	
		M	5	5.73	8	5.73	1	2.55	
		F	4	3.27	1	3.27	3	1.45	
5	IIIL-1		ss		si		ii	1.89	> 0.05
			Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	
		M	9	7.44	4	4.38	1	2.19	
		F	8	9.56	6	5.63	4	2.81	
	IIIL-3		ss		si		ii	4.91	> 0.05
			Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	
		M	7	9.19	7	4.38	0	0.44	
		F	14	11.81	3	5.63	1	0.56	

Contingency Analysis

Six inversions were dependent on site: IIL-6 (χ^2 value = 129.00; $p < 0.0001$), IIL-7 (χ^2 value = 129.00; $p < 0.0001$), IIIL-1 (χ^2 value = 47.58; $p = 0.0002$), IIIL-6 (χ^2 value = 138.82; $p < 0.0001$), IIIL-7 (χ^2 value = 119.87; $p < 0.0001$), and IIIL-9 (χ^2 value = 129.00; $p < 0.0001$). Other inversions (IL-1, IL-2, IIL-1, IIL-2, IIL-3, IIL-4, IIL-5, IIL-8, IIL-9, IIIL-2, IIIL-3, IIIL-4, IIIL-5, IIIL-8) were independent of site ($p > 0.0025$; see Table A.1 for all χ^2 values and p-values). Pairwise comparisons for inversions IIIL-1, IIIL-6, and IIIL-7 revealed possible structure within populations of cytoform A (Fig. 20). Pairwise comparisons of populations for inversion IIL-6 revealed populations 7 and 8 (*S. johannseni* cytoform B) were different from all other populations. Pairwise comparisons of sites for inversion IIL-7 showed populations 7, 8, 9, and 10 (*S. johannseni* cytoform B and *S. parmatum*) were different from *S. johannseni* cytoform A. Pairwise comparisons of inversion IIIL-1 resulted in two groups: populations 1, 2, 3, 4, 5, 6, 7, 9, and 10; and populations 2, 7, 8, 9, and 10. Pairwise comparisons of inversion IIIL-6 separated cytoform A from *S. parmatum* and cytoform B, with the exception of population 6, which was not significantly different from either grouping. Pairwise comparisons of inversion IIIL-7 divided populations 1, 2, 3, 4, 5, 6, 9, and 10 from populations 2, 4, 7, and 8. Pairwise comparisons of inversion IIIL-9 separated the *S. parmatum* samples from all *S. johannseni* samples.

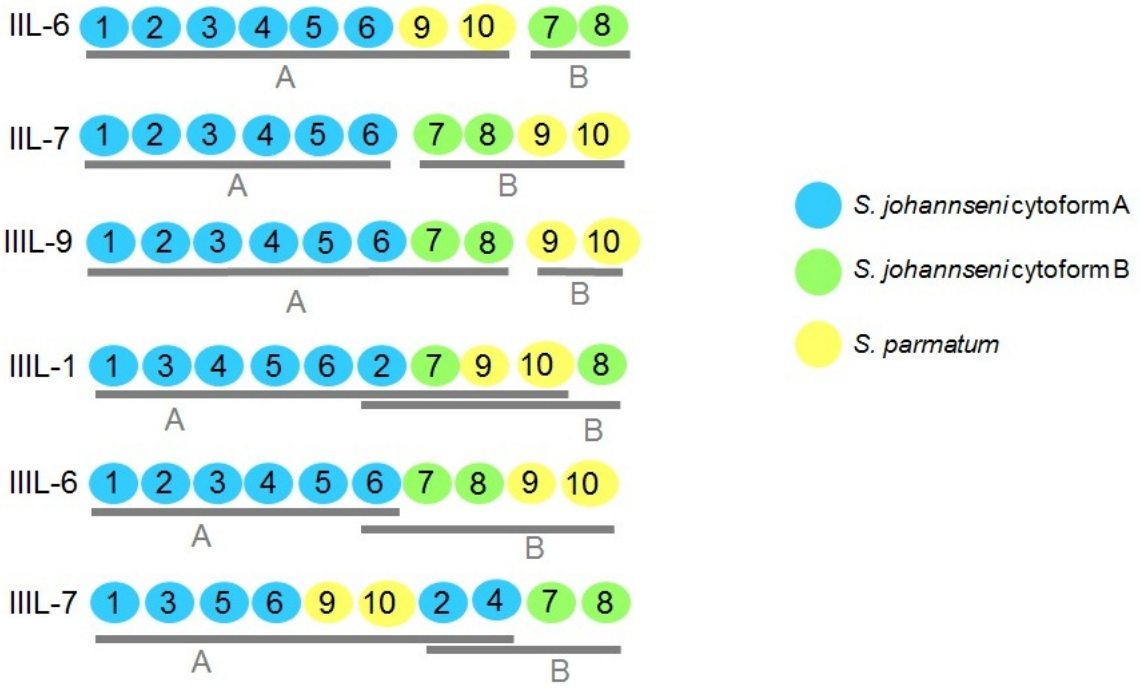

Fig. 20. Results of pairwise comparisons of populations for inversions dependent on site. Wisconsin populations, *S. johannseni* cytoform A, are blue; southern populations, *S. johannseni* cytoform B, are green; populations of *S. parmatum* are yellow. Inversion tested is labeled at the left of each row. Populations are labeled by site number. Gray bars group populations by significance – populations above the bar labeled "A" within an inversion are not significantly different from each other, but are significantly different from populations above the bar labeled "B." Significance was not tested between different inversions; "A" in one row does not correspond with "A" in a different row.

Assignment Test

A majority of larvae, 55.8%, were assigned to populations other than their population of origin, but were assigned to their region or species of origin (Table 7). Larvae of *S. johannseni* cytoform A (populations 1-6) were all assigned to either their own or another population from Wisconsin. Larvae of *S. johannseni* cytoform B (populations 7, 8) were assigned to South Carolina sites. *Simulium parmatum* larvae (populations 9, 10) were assigned to populations of *S. parmatum*.

Table 7. Assignment test results of multilocus genotype for 129 individuals of the *Simulium johannseni* group from 10 populations. Numbers in cells represent the number of larvae from a source population (left column) assigned to a particular population based on average genotype frequencies for each population. Bolded values represent larvae assigned to their actual source population. Appendix B gives log-likelihood assignment results for each individual.

Source	Assigned Population									
Population	1	2	3	4	5	6	7	8	9	10
1	**9**	2	2	5	2	2				
2		**3**		2						
3		3	**5**	3						
4				**5**						
5	4	3	5	11	**5**	4				
6				4		**2**				
7							**9**	1		
8							12	**10**		
9									**7**	2
10									5	**2**

Pairwise F_{ST} Comparison

Population pairwise F_{ST} values grouped *S. johannseni* cytoform A, *S. johannseni* cytoform B, and *S. parmatum* samples with each other, supporting the divisions delineated in the assignment test (Table 8). Populations of cytoform A (1-6) had low F_{ST} values when compared to each other relative to when they were compared with populations of cytoform B (7, 8) or *S. parmatum* (9, 10). Populations of cytoform B had a low, non-significant average F_{ST} value (0.03689) when compared with each other, but highly significant F_{ST} values when compared with populations of cytoform A or *S. parmatum*. Populations of *S. parmatum* had a non-significant average F_{ST} value of -0.06288 when compared with each other and highly significant F_{ST} values indicating significant differentiation from both forms of *S. johannseni*.

Table 8. Population pairwise F_{ST} values and p-values for all sampled populations. F_{ST} values are above the diagonal; p-values are below the diagonal. Bolded F_{ST} values have p-values < 0.05. See Appendix B for full F_{ST} values and exact p-values.

	1	2	3	4	5	6	7	8	9	10
1		0.00	0.02	-0.02	0.01	0.01	**0.74**	**0.74**	**0.70**	**0.69**
2	0.45		-0.02	-0.03	-0.02	0.05	**0.85**	**0.81**	**0.85**	**0.83**
3	0.17	0.83		-0.01	-0.02	-0.01	**0.73**	**0.74**	**0.69**	**0.66**
4	0.81	0.70	0.70		-0.02	-0.01	**0.87**	**0.82**	**0.88**	**0.86**
5	0.31	0.86	0.96	0.85		-0.00	**0.72**	**0.72**	**0.67**	**0.66**
6	0.50	0.13	0.50	0.29	0.65		**0.82**	**0.80**	**0.81**	**0.79**
7	0.00	0.00	0.00	0.00	0.00	0.00		0.04	**0.89**	**0.88**
8	0.00	0.00	0.00	0.00	0.00	0.00	0.23		**0.81**	**0.80**
9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		-0.06
10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.99	

Evolutionary Relationships

A phenogram was constructed using a neighbor-joining method based on Nei's standard genetic distance between the populations (Fig. 21). The tree has *S. johannseni* cytoform B and *S. parmatum* as less distant from each other than either population is from *S. johannseni* cytoform A. This result corroborates the cytodendrogram constructed based on shared inversions among the populations (Fig. 22). The most parsimonious tree is one in which *S. johannseni* cytoform B and *S. parmatum* are more closely related to each other than either population is to *S. johannseni* cytoform A. The hypothetical ancestral sequence is represented by the chosen standard in this study.

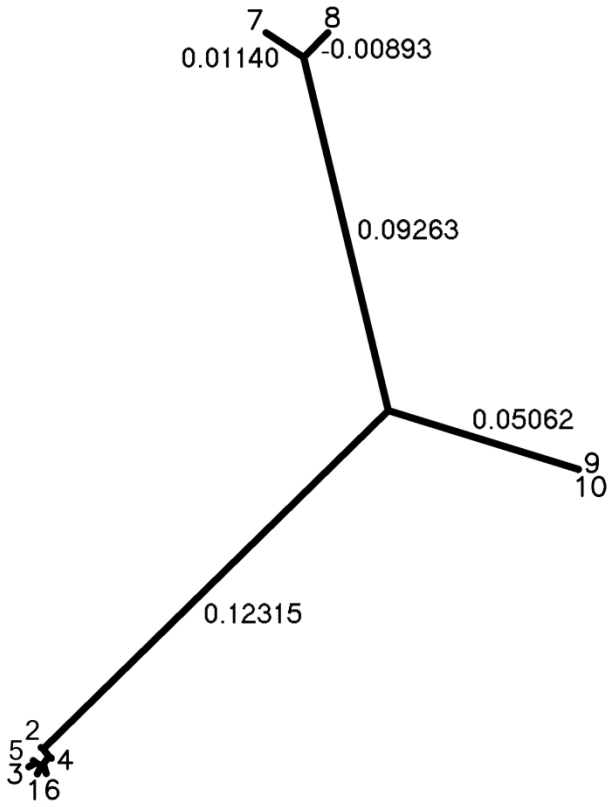

Fig. 21. Neighbor-joining phenogram of all populations. Populations 7 and 8 are *S. johannseni* cytoform B, populations 9 and 10 are *S. parmatum*, and populations 1-6 are *S. johannseni* cytoform A. Nei's standard genetic distance was calculated between all pairs of populations. Branches are to scale. Appendix D gives expanded phenogram with all branch lengths.

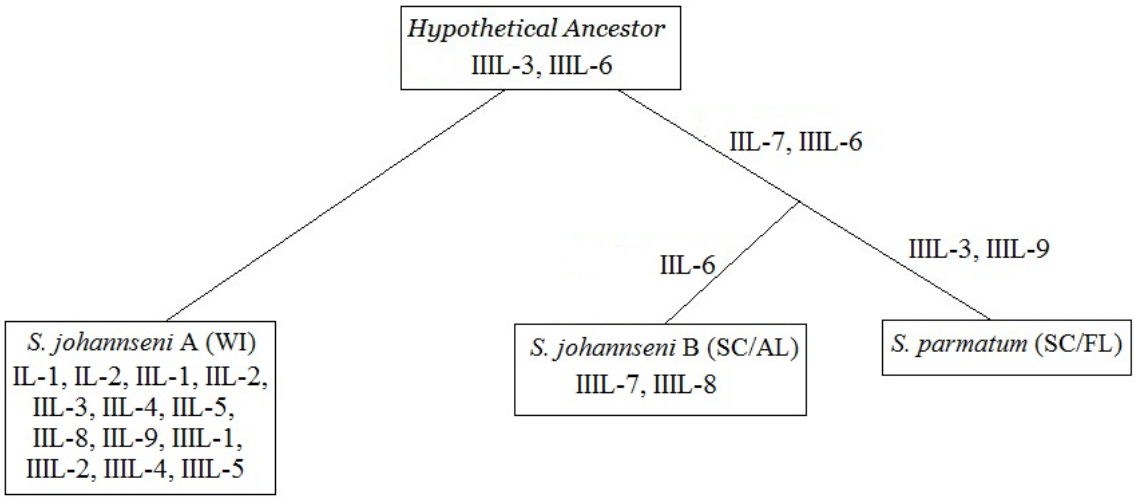

Fig. 22. Cytodendrogram of *S. johannseni* and *S. parmatum*. Inversions listed inside boxes are polymorphic within that cytoform. These inversions remain polymorphic in successive cytoforms unless otherwise listed. Inversions listed next to connecting lines are fixed in successive cytoforms. Inversion IIIL-3 in *S. parmatum* is possibly Y-linked. The hypothetical ancestor is based on the chosen standard banding pattern for each chromosome arm.

Discussion

Cryptic diversity exists within *Simulium johannseni*. Northern and southern populations of *S. johannseni* can be separated into two cytoforms, but more evidence is needed to determine whether these cytoforms are separate species or cytotypes of a single species. The lack of population structure between Wisconsin populations, as shown by the high level of mis-assignment between sampling sites in the assignment test, non-significant F_{ST} values, and small genetic distances between populations, indicates one interbreeding population in Wisconsin. This result does not support the hypothesized existence of a cryptic population of flies specializing on whooping cranes within the Wisconsin population. The Wisconsin cytoform (A) of *S. johannseni* might be

specialized for feeding on whooping cranes relative to cytoform B; thus the entire population of Wisconsin *S. johannseni* should be managed as a pest of whooping cranes. The grouping of cytoform A as one interbreeding population is further supported by the pairwise comparisons of populations, in which cytoform A populations are not significantly different from each other based on chi-square analysis. Inversions IIIL-1, IIIL-6, and IIIL-7 group some cytoform A populations with cytoform B or *S. parmatum*, but the possibility of population structure within Wisconsin based on the chi-square pairwise comparisons might be an artifact of small sample sizes, as it is contradicted by the genotype assignment test results, F_{ST} values, and genetic distances between the Wisconsin populations.

Chromosomal polymorphism in species groups of black flies varies widely (Adler et al. 2004). Species groups have been reported with seven fixed and five polymorphic inversions in one pair of siblings and one fixed and over 100 polymorphic inversions in a closely related pair of sibling species (Bedo 1977). Species groups have been reported with relatively little variation, as well; for example, the *Eusimulium* (now *Simulium*) *canonicolum* group features only two fixed inversion differences between four of its members, and around 30 polymorphic inversions among group members examined (Golini and Rothfels 1984). Some species are monomorphic, meaning they contain no polymorphic inversions and every individual shares the exact same banding pattern (Adler et al. 2004).

Cytoform A (Wisconsin) and cytoform B (Alabama and South Carolina) of *S. johannseni* are differentiated by fixed inversions IIL-6, IIL-7, and IIIL-6 and a high

frequency of IIIL-7 in southern populations. These differences might indicate separate species, but alternatively they might be extremes in clinal variation. The shortest straight-line distance between the populations, between site 5 (River Road, Lemonweir River, WI) and site 8 (Murder Creek, AL), is approximately 1460 km; site 7 (Rt. 377, Black River, SC) is slightly farther from site 5 at approximately 1466 km (Table 2). Black flies have been known to disperse up to 35 km, but generally remain within 9 to 13 km from their emergence site (Baldwin et al. 1975), so the probability of Wisconsin and southern populations of *S. johannseni* interbreeding is low. Further study of populations from sites between Wisconsin and South Carolina and Alabama should provide insight into whether these inversions define a separate species or vary based on environmental variation.

Where *S. johannseni* and *S. parmatum* are sympatric, morphological variation can impede species identification. *Simulium parmatum* typically has a very dark, shield-shaped marking on its head capsule, whereas *S. johannseni* typically has a light head capsule with a pattern of darker spots (Adler et al. 2004). In some instances, however, *S. johannseni* larvae may have a dark head capsule that obscures the typical spot pattern and makes the larvae appear to be *S. parmatum*. Three *S. johannseni* larvae that I analyzed from the Black River, SC site (site 7), featured a dark head pattern and were initially identified as *S. parmatum*. Chromosomal analysis resolved this issue, and re-examination of the morphology of the misidentified larvae confirmed that they were *S. johannseni*, not *S. parmatum*. In chromosome arm IIL, *S. johannseni* is fixed for inversions IIL-6 and IIL-7, whereas *S. parmatum* is fixed for inversion IIL-7. *Simulium parmatum* is fixed for inversion IIIL-9, which is easily distinguished from the distal end of IIIL in *S. johannseni*

cytoform B, even though both *S. parmatum* and *S. johannseni* cytoform B are fixed for inversion IIIL-6.

The characteristics of habitat available to black flies vary within ecological regions, or ecoregions, throughout North America (Commission for Environmental Cooperation Working Group [CECWG] 1997). All sites sampled in Wisconsin fall within ecoregion 8.1, mixed woods plains, whereas the South Carolina site lies in ecoregion 8.5, the Mississippi alluvian and southeast U.S.A. coastal plains, and the Alabama site lies within ecoregion 8.3, the southeastern U.S.A. plains. These are all subdivisions of ecoregion 8, the eastern temperate forests, which can be broadly characterized as having a temperate climate with many deciduous tree species growing in soil that ranges from nutrient-poor to calcium-rich (CECWG 1997). Chromosomal inversion polymorphisms have been correlated with environmental variation in previous work with Diptera (Sharakhova et al. 2011, White et al. 2011, Michailova et al. 2012), so it is not unreasonable to infer that variation within the *S. johannseni* group is also correlated with environmental variation. Within black flies, temperature is known to influence the extent of pairing between homologous chromosomes (Rothfels and Featherston 1981). Future work could investigate whether inversions in the *S. johannseni* group are correlated with variation in rainfall, stream chemistry, soil chemistry, or types of organic matter. Cytological variation could also be correlated with environmental degradation. In chironomid larvae, heavy metal pollution of streams was associated with fixed inversion differences between species as well as chromosomal rearrangements and puffing of DNA in individual larvae (Michailova et al. 2012). In black flies, degraded water quality,

including the presence of heavy metals, can influence chromosome structure and reflect change in the number of B chromosomes present (Sanderson et al. 1982, Kachvoryan et al. 1996). Black fly larvae in Ghana that carry certain inversions are resistant to some pesticides; these inversions can be used to track the spread of resistance through populations (Osei-Atweneboana et al. 2001). Cytological changes that correlate with the presence of environmental stressors allow the use of larvae as genosensors, allowing for monitoring of stream quality by examining larval chromosomes.

Based on the ecoregions of my collecting sites, I hypothesize that known populations of *S. johannseni* in the Southeast, such as those reported in Texas and Georgia (Adler et al. 2004) in ecoregion 8.3, would have inversion frequencies similar to cytoform B, whereas populations of *S. johannseni* in Missouri and Illinois would have inversion frequencies more similar to cytoform A. Sampling of probable *S. johannseni* larval habitat between these areas, such as in Tennessee, Arkansas, and northern Mississippi and Georgia, could shed additional light on polymorphic variation and reveal whether the currently identified polymorphisms represent different species or clinal variation. Another direction for future work could investigate more western populations of *S. johannseni*. Populations reported from central Iowa and areas north and west, including reported populations in Kansas, Nebraska, and Canada (Adler et al. 2004), fall within ecoregion 9, the Great Plains, which is drier than the eastern temperate forest and is dominated by grass rather than trees (CECWG 1997). The intense temperature fluctuations between summer and winter in this region might act as a selective force and result in differentiation in chromosomal polymorphisms. Chromosomal analysis of these

populations might shed light on the status of *S. johannseni duplex*, which is most commonly found in the northern part of the *S. johannseni* range (Adler et al. 2004). Currently, *S. johannseni duplex* is considered to be a morphological variant of *S. johannseni* in which pupae have two gill filaments instead of four (Adler et al. 2004); chromosomal analysis would reveal whether or not the morphological variation corresponds with chromosomal variation, ultimately permitting identification of *S. johannseni duplex* as a separate species or an intraspecific variant of *S. johannseni*.

Sampling of additional populations of *S. johannseni* could provide insight into evolutionary relationships as well. Older populations have had more time to accumulate polymorphisms; I hypothesize that *S. johannseni* cytoform A is closer to the ancestral form as represented by my chosen standard banding pattern. One possible explanation for the chromosomal differences between *S. johannseni* cytoform A and *S. johannseni* cytoform B and *S. parmatum* is that a small founding population with a few polymorphisms dispersed from the mixed woods plains ecoregion southward, into the southeastern plains and coastal plains regions, where it acquired fixed inversions that provided a selective advantage in the new environment. In this case, the southern populations of *S. johannseni*, with their closer relationship to *S. parmatum* than to northern populations of *S. johannseni*, might represent a separate species that has secondarily acquired morphology (i.e., head capsule markings) similar to northern *S. johannseni*. This interpretation would change if some inversions identified here are actually the ancestral, rather than derived, sequence for the group. For example, if inversions IIL-7 and IIIL-6, which were interpreted as uniting cytoform B and *S.*

parmatum into a clade based on the current standard map, are actually the standard, then each group of flies studied would be characterized by one fixed inversion: cytoform A would be characterized by inversion IIL-7 (currently the standard), cytoform B would be characterized by inversion IIL-6 (as in the current interpretation), and *S. parmatum* would be characterized by inversion IIIL-9 (as in the current interpretation). In this case, the three populations would form a trichotomy. Ultimately, an outgroup is necessary to determine the true standard (i.e., ancestral) sequence and evolutionary relationships within the group.

Currently, the *S. johannseni* group is considered monophyletic based on morphology (Adler et al. 2004). To fully understand chromosomal rearrangements within the *S. johannseni* clade, comparisons must be made to a closely related species or group of species outside the group, such as *Simulium canonicolum*, which is in the same subgenus as *S. johannseni*, *Boreosimulium*, but in a different species group (Golini and Rothfels 1984). Banding patterns shared between these two groups can be inferred to be inherited from the common ancestor of the groups. *Simulium canonicolum* shares banding patterns at the ends of IS, IIS, the distal three-fourths of IIIS, and near the centromere in IIL (Golini and Rothfels 1984). Several landmarks are in similar positions, including the basal marker in IIIL, suggesting that my chosen standard orientation of IIIL-1 is ancestral, and the trapezoidal and Ring of Balbiani in IIS (Golini and Rothfels 1984). These similarities suggest that my chosen standard might be, for some sections of some arms, a reasonable hypothesis of the ancestral banding pattern.

Summary

1. *Simulium johannseni* can be divided into two cytoforms. Cytoform A has fifteen polymorphic inversions and no fixed inversions and is found in Wisconsin. Cytoform B has three polymorphic and three fixed inversions and is found near the coastal plain in the southeastern U.S.
2. Management of *S. johannseni* in Wisconsin as a pest of whooping cranes should consider all local populations as one large, interbreeding population of a single species.
3. Based on shared fixed inversions and Nei's genetic distance, *S. parmatum* is more closely related to *S. johannseni* cytoform B than either is to *S. johannseni* cytoform A.
4. Further study of additional populations is needed to determine the status of the *S. johannseni* cytoforms, the possible correlation of inversions with environmental variation, and the evolutionary history of the *S. johannseni* group.

APPENDICES

Appendix A

Chi-square Contingency Tests

Table A.1. Results of χ^2 test for independence. Significant inversions (p-value ≤ 0.0025 after Bonferroni correction) are bolded.

Inversion	χ^2 Value	p-value	Significant?
IL-1	11.6349	0.8655	No
IL-2	14.1343	0.7203	No
IIL-1	14.9383	0.0926	No
IIL-2	14.7652	0.6780	No
IIL-3	20.0815	0.3283	No
IIL-4	8.0565	0.5285	No
IIL-5	11.2727	0.8824	No
IIL-6	**129.0000**	**< 0.0001**	**Yes**
IIL-7	**129.0000**	**< 0.0001**	**Yes**
IIL-8	4.9016	0.8428	No
IIL-9	4.3076	0.8900	No
IIIL-1	**47.5812**	**0.0002**	**Yes**
IIIL-2	30.2508	0.0351	No
IIIL-3	19.8965	0.3387	No
IIIL-4	21.9002	0.2364	No
IIIL-5	10.8111	0.2889	No
IIIL-6	**138.8230**	**< 0.0001**	**Yes**
IIIL-7	**119.8695**	**< 0.0001**	**Yes**
IIIL-8	4.9016	0.8428	No
IIIL-9	**129.0000**	**< 0.0001**	**Yes**

Table A.2. Results of pairwise contingency test for inversion IIL-6. χ^2 values are above the diagonal, p-values are below the diagonal. Bonferroni corrected significance level is 0.001111. Comparisons were not made between populations with no variation in genotype frequencies; these cells are blank. All tested pairs were significantly different.

	1	2	3	4	5	6	7	8	9	10
1							32.00	44.00		
2							15.00	27.00		
3							21.00	33.00		
4							15.00	27.00		
5							42.00	54.00		
6							16.00	28.00		
7	<0.0001	0.0001	<0.0001	0.0001	<0.0001	<0.0001			19.00	17.00
8	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001			31.00	29.00
9							<0.0001	<0.0001		
10							<0.0001	<0.0001		

Table A.3. Results of pairwise contingency test for inversion IIL-7. χ^2 values are above the diagonal, p-values are below the diagonal. Bonferroni corrected significance level is 0.001111. Comparisons were not made between populations with no variation in genotype frequencies; these cells are blank. All tested pairs were significantly different.

	1	2	3	4	5	6	7	8	9	10
1							32.00	44.00	31.00	29.00
2							15.00	27.00	14.00	12.00
3							21.00	33.00	20.00	18.00
4							15.00	27.00	14.00	12.00
5							42.00	54.00	41.00	39.00
6							16.00	28.00	15.00	13.00
7	<0.0001	0.0001	<0.0001	0.0001	<0.0001	<0.0001				
8	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001				
9	<0.0001	0.0002	<0.0001	0.0002	<0.0001	0.0001				
10	<0.0001	0.0005	<0.0001	0.0005	<0.0001	0.0003				

Table A.4. Results of pairwise contingency test for inversion IIIL-1. χ^2 values are above the diagonal, p-values are below the diagonal. Bonferroni corrected significance level is 0.001111. Comparisons were not made between populations with no variation in genotype frequencies; these cells are blank. Significantly different pairs are bolded.

	1	2	3	4	5	6	7	8	9	10
1		1.24	0.47	1.24	0.80	1.46	9.95	**18.45**	9.16	7.50
2	0.5369		0.62	0.40	0.92	2.60	4.62	9.50	4.20	3.36
3	0.7900	0.7322		0.62	0.82	1.43	7.64	**14.67**	7.01	5.73
4	0.5369	0.5271	0.7322		1.94	1.40	7.50	**14.85**	6.87	5.60
5	0.6700	0.6309	0.6643	0.3784		3.14	7.29	**14.28**	6.65	5.33
6	0.4830	0.2729	0.4890	0.4974	0.2079		12.12	**22.32**	11.25	9.48
7	0.0069	0.0317	0.0220	0.0062	0.0261	0.0023				
8	**<0.0001**	0.0021	**0.0007**	**0.0001**	**0.0008**	**<0.0001**				
9	0.0103	0.0404	0.0300	0.0088	0.0359	0.0036				
10	0.0236	0.0668	0.0571	0.0180	0.0695	0.0087				

Table A.5. Results of pairwise contingency test for inversion IIIL-6. χ^2 values are above the diagonal, p-values are below the diagonal. Bonferroni corrected significance level is 0.001111. Comparisons were not made between populations with no variation in genotype frequencies; these cells are blank. Significantly different pairs are bolded.

	1	2	3	4	5	6	7	8	9	10
1					0.70	3.80	**32.00**	**44.00**	**31.00**	**29.00**
2					0.16	0.92	**15.00**	**27.00**	**14.00**	**12.00**
3					0.35	1.95	**21.00**	**33.00**	**20.00**	**18.00**
4					0.16	0.92	**15.00**	**27.00**	**14.00**	**12.00**
5	0.4026	0.6886	0.5530	0.6886		1.86	**42.00**	**54.00**	**41.00**	**39.00**
6	0.0512	0.3384	0.1628	0.3384	0.1728		**16.00**	**28.00**	**15.00**	13.00
7	**<0.0001**	**0.0001**	**<0.0001**	**0.0001**	**<0.0001**	**0.0003**				
8	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**				
9	**<0.0001**	**0.0002**	**<0.0001**	**0.0002**	**<0.0001**	**0.0006**				
10	**<0.0001**	**0.0005**	**<0.0001**	**0.0005**	**<0.0001**	0.0015				

Table A.6. Results of pairwise contingency test for inversion IIIL-7. χ^2 values are above the diagonal, p-values are below the diagonal. Bonferroni corrected significance level is 0.001111. Comparisons were not made between populations with no variation in genotype frequencies; these cells are blank. Significantly different pairs are bolded.

	1	2	3	4	5	6	7	8	9	10
1							**27.55**	**30.46**		
2							**11.25**	12.27		
3							**17.33**	**19.80**		
4							**11.25**	12.27		
5							**36.65**	**39.27**		
6							**12.34**	**13.75**		
7	**<0.0001**	**0.0008**	**<0.0001**	**0.0008**	**<0.0001**	**0.0004**		4.34	**15.39**	**13.39**
8	**<0.0001**	0.0022	**<0.0001**	0.0022	**<0.0001**	**0.0010**	0.1143		**17.56**	**15.10**
9							**<0.0001**	**0.0002**		
10							**0.0003**	**0.0005**		

Table A.7. Results of pairwise contingency test for inversion IIIL-9. χ^2 values are above the diagonal, p-values are below the diagonal. Bonferroni corrected significance level is 0.001111. Comparisons were not made between populations with no variation in genotype frequencies; these cells are blank. All tested pairs were significantly different.

	1	2	3	4	5	6	7	8	9	10
1									31.00	29.00
2									14.00	12.00
3									20.00	18.00
4									14.00	12.00
5									41.00	39.00
6									15.00	13.00
7									19.00	17.00
8									31.00	29.00
9	<0.0001	0.0002	<0.0001	0.0002	<0.0001	0.0001	<0.0001	<0.0001		
10	<0.0001	0.0005	<0.0001	0.0005	<0.0001	0.0003	<0.0001	<0.0001		

Appendix B

Results of Genotype Assignment Test

Table B.1. Assignment test results for population 1. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
1	**-3.204**	-6.589	-4.347	-5.871	-3.790	-3.495	-51.282	-55.283	-45.745	-43.811
2	**-7.484**	-10.984	-20.688	-16.422	-14.390	-17.469	-63.216	-68.793	-57.469	-55.033
3	**-5.331**	-10.173	-11.172	-11.994	-7.286	-8.953	-63.216	-68.793	-57.469	-55.033
4	**-8.194**	-12.676	-11.326	-15.036	-8.886	-13.024	-61.830	-67.407	-56.083	-53.647
5	**-4.360**	-5.591	-10.019	-7.412	-6.034	-7.248	-57.942	-62.731	-52.300	-50.115
6	**-4.154**	-6.589	-8.564	-5.871	-8.974	-6.555	-51.282	-55.283	-45.745	-43.811
7	-6.272	-11.172	-6.698	-10.453	**-5.805**	-8.259	-56.556	-61.345	-50.914	-48.729
8	-5.530	-5.896	-4.278	-5.717	**-4.017**	-7.248	-46.008	-49.221	-40.576	-38.893
9	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
10	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
11	-5.309	-5.591	-5.180	-4.333	-4.792	**-2.889**	-53.709	-57.822	-48.518	-46.296
12	**-6.534**	-14.062	-12.255	-16.422	-8.444	-11.350	-63.216	-68.793	-57.469	-55.033
13	-5.530	**-2.007**	-3.936	-5.717	-3.695	-7.248	-46.008	-49.221	-40.576	-38.893
14	-7.222	-8.093	**-6.076**	-7.375	-6.760	-6.960	-52.323	-56.436	-47.131	-44.909
15	-8.551	-11.865	-9.214	**-5.529**	-16.701	-13.398	-52.668	-56.669	-47.131	-45.197
16	-3.434	**-2.818**	-8.495	-5.717	-4.457	-7.248	-46.008	-49.221	-40.576	-38.893
17	**-4.384**	-5.896	-8.495	-5.717	-8.878	-7.248	-46.008	-49.221	-40.576	-38.893
18	-7.692	-11.865	**-6.817**	-11.685	-7.858	-13.398	-52.668	-56.669	-47.131	-45.197
19	-7.452	-7.400	-9.025	**-7.221**	-9.651	-7.653	-47.049	-50.374	-41.963	-39.991
21	-3.388	-4.087	-4.650	-2.829	-4.019	**-2.484**	-52.668	-56.669	-47.131	-45.197
22	-2.462	-1.314	-3.125	**-1.135**	-2.442	-2.484	-40.733	-43.159	-35.408	-33.975
24	**-5.792**	-11.865	-6.817	-11.685	-6.334	-7.280	-52.668	-56.669	-47.131	-45.197

Table B.2. Assignment test results for population 2. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
1	-5.530	**-2.007**	-3.936	-5.717	-3.695	-7.248	-46.008	-49.221	-40.576	-38.893
2	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
3	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
4	-5.356	**-4.322**	-9.025	-7.221	-5.230	-7.653	-47.049	-50.374	-41.963	-39.991
5	-5.530	**-2.007**	-3.936	-5.717	-3.695	-7.248	-46.008	-49.221	-40.576	-38.893

Table B.3. Assignment test results for population 3. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
1	-8.378	-6.284	**-5.991**	-8.916	-6.045	-7.653	-58.983	-63.884	-53.687	-51.214
2	-5.300	**-2.700**	-4.005	-5.871	-3.790	-6.555	-51.282	-55.283	-45.745	-43.811
3	-4.583	-6.589	-5.546	**-2.793**	-8.974	-6.555	-51.282	-55.283	-45.745	-43.811
4	-2.462	-1.314	-3.125	**-1.135**	-2.442	-2.484	-40.733	-43.159	-35.408	-33.975
5	-5.530	-5.896	**-5.477**	-5.717	-5.892	-7.248	-46.008	-49.221	-40.576	-38.893
6	-5.530	**-2.007**	-3.936	-5.717	-3.695	-7.248	-46.008	-49.221	-40.576	-38.893
7	-7.452	-10.478	-5.888	**-10.299**	-6.776	-8.953	-51.282	-55.283	-45.745	-43.811
8	-5.530	**-2.007**	-3.936	-5.717	-3.695	-7.248	-46.008	-49.221	-40.576	-38.893
9	-19.090	-32.085	**-14.178**	-31.367	-16.353	-24.462	-78.620	-85.994	-73.352	-69.839
10	-14.570	-20.148	**-10.755**	-19.430	-11.057	-12.161	-65.299	-71.098	-60.242	-57.230
11	-15.816	-17.140	**-9.237**	-16.422	-13.511	-17.469	-63.216	-68.793	-57.469	-55.033

Table B.4. Assignment test results for population 4. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
1	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
2	-2.462	-1.314	-3.125	**-1.135**	-2.442	-2.484	-40.733	-43.159	-35.408	-33.975
3	-4.583	-6.589	-5.546	**-2.793**	-8.974	-6.555	-51.282	-55.283	-45.745	-43.811
4	-4.384	-2.818	-3.656	**-2.639**	-3.215	-2.889	-41.775	-44.312	-36.794	-35.073
5	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893

Table B.5. Assignment test results for population 5. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
1	-5.309	-5.591	-5.180	-4.333	-4.792	**-2.889**	-53.709	-57.822	-48.518	-46.296
2	-11.907	-13.369	-8.261	-13.190	-9.240	**-7.685**	-53.709	-57.822	-48.518	-46.296
3	-19.470	-27.961	**-15.041**	-34.481	-17.219	-29.108	-88.471	-97.199	-82.303	-78.863
4	**-6.328**	-8.904	-10.178	-11.803	-6.483	-9.358	-52.323	-56.436	-47.131	-44.909
5	-9.524	-9.362	**-7.070**	-11.994	-7.591	-8.953	-63.216	-68.793	-57.469	-55.033
6	-4.384	-2.818	-3.656	**-2.639**	-3.215	-2.889	-41.775	-44.312	-36.794	-35.073
7	-10.760	-10.291	-7.924	-10.111	**-7.389**	-9.445	-49.477	-52.913	-44.735	-42.476
8	**-3.204**	-3.511	-8.564	-5.871	-4.552	-6.555	-51.282	-55.283	-45.745	-43.811
9	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
10	**-3.204**	-3.511	-8.564	-5.871	-4.552	-6.555	-51.282	-55.283	-45.745	-43.811
11	-5.300	**-2.700**	-4.005	-5.871	-3.790	-6.555	-51.282	-55.283	-45.745	-43.811
12	-4.384	-2.818	-3.656	**-2.639**	-3.215	-2.889	-41.775	-44.312	-36.794	-35.073
13	-2.462	-1.314	-3.125	**-1.135**	-2.442	-2.484	-40.733	-43.159	-35.408	-33.975
14	-2.462	-1.314	-3.125	**-1.135**	-2.442	-2.484	-40.733	-43.159	-35.408	-33.975
15	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
16	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
17	-11.907	**-5.591**	-6.664	-13.190	-7.107	-13.804	-53.709	-57.822	-48.518	-46.296
18	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
19	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
20	-10.520	-11.982	-7.160	-11.803	**-6.806**	-12.417	-52.323	-56.436	-47.131	-44.909
21	-5.530	-5.896	**-5.477**	-5.717	-5.892	-7.248	-46.008	-49.221	-40.576	-38.893
22	-8.524	-5.896	-8.495	-5.717	-5.892	**-4.188**	-34.073	-35.711	-28.852	-27.671
23	-5.300	**-2.700**	-4.005	-5.871	-3.790	-6.555	-51.282	-55.283	-45.745	-43.811
24	-5.309	-5.591	-5.180	-4.333	-4.792	**-2.889**	-53.709	-57.822	-48.518	-46.296
25	-17.308	-25.881	**-12.844**	-28.513	-13.701	-22.958	-81.811	-89.751	-75.748	-72.559
26	-4.384	-2.818	-3.656	**-2.639**	-3.215	-2.889	-41.775	-44.312	-36.794	-35.073
27	-16.209	-25.424	-16.409	-28.322	**-13.659**	-23.364	-70.918	-77.394	-65.411	-62.436
28	-8.599	-10.478	-6.629	-10.299	**-6.032**	-12.012	-51.282	-55.283	-45.745	-43.811
29	-7.692	-11.865	**-6.817**	-11.685	-7.858	-13.398	-52.668	-56.669	-47.131	-45.197
30	-5.530	-5.896	-5.477	-5.717	**-4.457**	-7.248	-46.008	-49.221	-40.576	-38.893
31	**-3.434**	-5.896	-4.278	-5.717	-3.695	-4.188	-46.008	-49.221	-40.576	-38.893
32	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893

Table B.6. Assignment test results for population 6. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
1	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
2	-9.349	-14.755	-7.942	-13.498	-8.364	**-6.299**	-64.257	-69.946	-58.855	-56.131
3	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
4	-4.384	-2.818	-3.656	**-2.639**	-3.215	-2.889	-41.775	-44.312	-36.794	-35.073
5	-2.232	-2.007	-3.194	**-1.289**	-2.537	-1.791	-46.008	-49.221	-40.576	-38.893
6	-10.216	-8.093	-9.094	-7.375	-6.760	**-3.901**	-40.389	-42.926	-35.408	-33.687

Table B.7. Assignment test results for population 7. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
9	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
11	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
18	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
22	-44.914	-34.470	-40.030	-34.291	-40.019	-29.514	-5.972	**-3.782**	-25.070	-23.851
24	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
27	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
40	-58.424	-45.021	-52.155	-44.842	-54.278	-40.428	**-1.577**	-2.258	-36.794	-35.073
2	-58.424	-45.021	-52.155	-44.842	-54.278	-40.428	**-1.577**	-2.258	-36.794	-35.073
8	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
12	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975

Table B.8. Assignment test results for population 8. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
1	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-4.930	**-2.629**	-23.684	-22.753
2	-58.424	-45.021	-52.155	-44.842	-54.278	-40.428	**-1.577**	-2.258	-36.794	-35.073
3	-49.054	-37.548	-44.869	-37.369	-45.682	-33.873	-2.040	**-1.174**	-28.852	-27.671
4	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
5	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
6	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
7	-49.054	-37.548	-44.869	-37.369	-45.682	-33.873	-2.040	**-1.174**	-28.852	-27.671
8	-49.054	-37.548	-44.869	-37.369	-45.682	-33.873	-2.040	**-1.174**	-28.852	-27.671
9	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
10	-50.976	-39.052	-45.400	-38.873	-46.456	-34.278	-3.081	**-2.327**	-30.239	-28.769
11	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
12	-44.914	-34.470	-40.030	-34.291	-40.019	-29.514	-5.972	**-3.782**	-25.070	-23.851
13	-58.424	-45.021	-52.155	-44.842	-54.278	-40.428	**-1.577**	-2.258	-36.794	-35.073
14	-58.424	-45.021	-52.155	-44.842	-54.278	-40.428	**-1.577**	-2.258	-36.794	-35.073
15	-49.054	-37.548	-44.869	-37.369	-45.682	-33.873	-2.040	**-1.174**	-28.852	-27.671
16	-58.424	-45.021	-52.155	-44.842	-54.278	-40.428	**-1.577**	-2.258	-36.794	-35.073
17	-49.054	-37.548	-44.869	-37.369	-45.682	-33.873	-10.204	**-5.698**	-28.852	-27.671
18	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
19	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975
20	-49.054	-37.548	-44.869	-37.369	-45.682	-33.873	-2.040	**-1.174**	-28.852	-27.671
21	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-4.930	**-2.629**	-23.684	-22.753
22	-56.502	-43.517	-51.625	-43.338	-53.505	-40.023	**-0.536**	-1.105	-35.408	-33.975

Table B.9. Assignment test results for population 9. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
3	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
5	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
6	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
7	-44.914	-34.470	-40.030	-34.291	-40.019	-29.514	-29.841	-30.802	-1.622	**-1.407**
9	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
10	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
11	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
14	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
15	-44.914	-34.470	-40.030	-34.291	-40.019	-29.514	-29.841	-30.802	-1.622	**-1.407**

Table B.10. Assignment test results for population 10. The likelihood of the most likely population for each individual (Ind.) is bolded.

	Assigned Population									
Ind.	1	2	3	4	5	6	7	8	9	10
1	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
2	-44.914	-34.470	-40.030	-34.291	-40.019	-29.514	-29.841	-30.802	-1.622	**-1.407**
3	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
4	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
5	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
6	-42.992	-32.966	-39.500	-32.787	-39.246	-29.108	-28.799	-29.649	**-0.236**	-0.308
7	-44.914	-34.470	-40.030	-34.291	-40.019	-29.514	-29.841	-30.802	-1.622	**-1.407**

Appendix C

Results of Pairwise F_{ST} Comparison

Table C.1. Population pairwise F_{ST} values.

	1	2	3	4	5	6	7	8	9	10
1	0.00000									
2	0.00452	0.00000								
3	0.02150	-0.01721	0.00000							
4	-0.01580	-0.03009	-0.00977	0.00000						
5	0.01086	-0.02333	-0.01912	-0.01674	0.00000					
6	0.00609	0.04704	-0.00589	-0.01341	-0.00362	0.00000				
7	0.74288	0.85225	0.72707	0.86780	0.71522	0.82455	0.00000			
8	0.74408	0.81499	0.73936	0.82360	0.71643	0.80109	0.03689	0.00000		
9	0.70278	0.85400	0.68823	0.87532	0.66906	0.81491	0.89137	0.80742	0.00000	
10	0.68774	0.83277	0.66246	0.85697	0.65642	0.79042	0.88029	0.79658	-0.06288	0.00000

Table C.2. Population pairwise F_{ST} p-values.

	1	2	3	4	5	6	7	8	9	10
1	*									
2	0.45045 ±0.05	*								
3	0.17117 ±0.03	0.82883 ±0.03	*							
4	0.81081 ±0.02	0.70270 ±0.04	0.70270 ±0.05	*						
5	0.30631 ±0.04	0.86486 ±0.02	0.96396 ±0.02	0.84685 ±0.04	*					
6	0.49550 ±0.05	0.12613 ±0.04	0.50450 ±0.04	0.28829 ±0.04	0.64865 ±0.04	*				
7	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	*			
8	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.23423 ±0.04	*		
9	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	*	
10	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.00000 ±0.00	0.99099 ±0.00	*

Appendix D

Tree Construction

Table D.1. Pairwise matrix of Nei's standard genetic distance (D_s).

	1	2	3	4	5	6	7	8	9	10
1	0									
2	0.005719	0								
3	0.006105	0.005538	0							
4	0.003651	0.003653	0.005825	0						
5	0.003044	0.003099	0.001238	0.003468	0					
6	0.005456	0.009743	0.006372	0.004832	0.004427	0				
7	0.239136	0.226023	0.241963	0.225298	0.233118	0.229494	0			
8	0.218127	0.205517	0.220993	0.204878	0.212369	0.20909	0.002464	0		
9	0.184315	0.173276	0.186846	0.17292	0.179074	0.176764	0.154106	0.134789	0	
10	0.184853	0.173637	0.186764	0.173272	0.179129	0.176552	0.154226	0.134945	5.02E-05	0

Table D.2. Branch lengths of dendrogram.
Refer to Fig. D.1 for phenogram structure.
n = node, p = population.

Between	And	Length
n7	n6	0.001592530000
n6	p3	0.002094020000
n6	p5	-0.000855565000
n7	p1	0.002363030000
n1	p7	0.011398000000
n1	p8	-0.008933980000
n1	n3	0.092634700000
n2	p9	-0.000056983700
n2	p10	0.000107222000
n2	n3	0.050624800000
n4	p2	-0.000792170000
n4	n3	0.123147000000
n4	n5	0.002329080000
n5	p4	-0.000763135000
n5	n8	0.002414730000
n8	p6	0.002494710000
n7	n8	0.000645645623

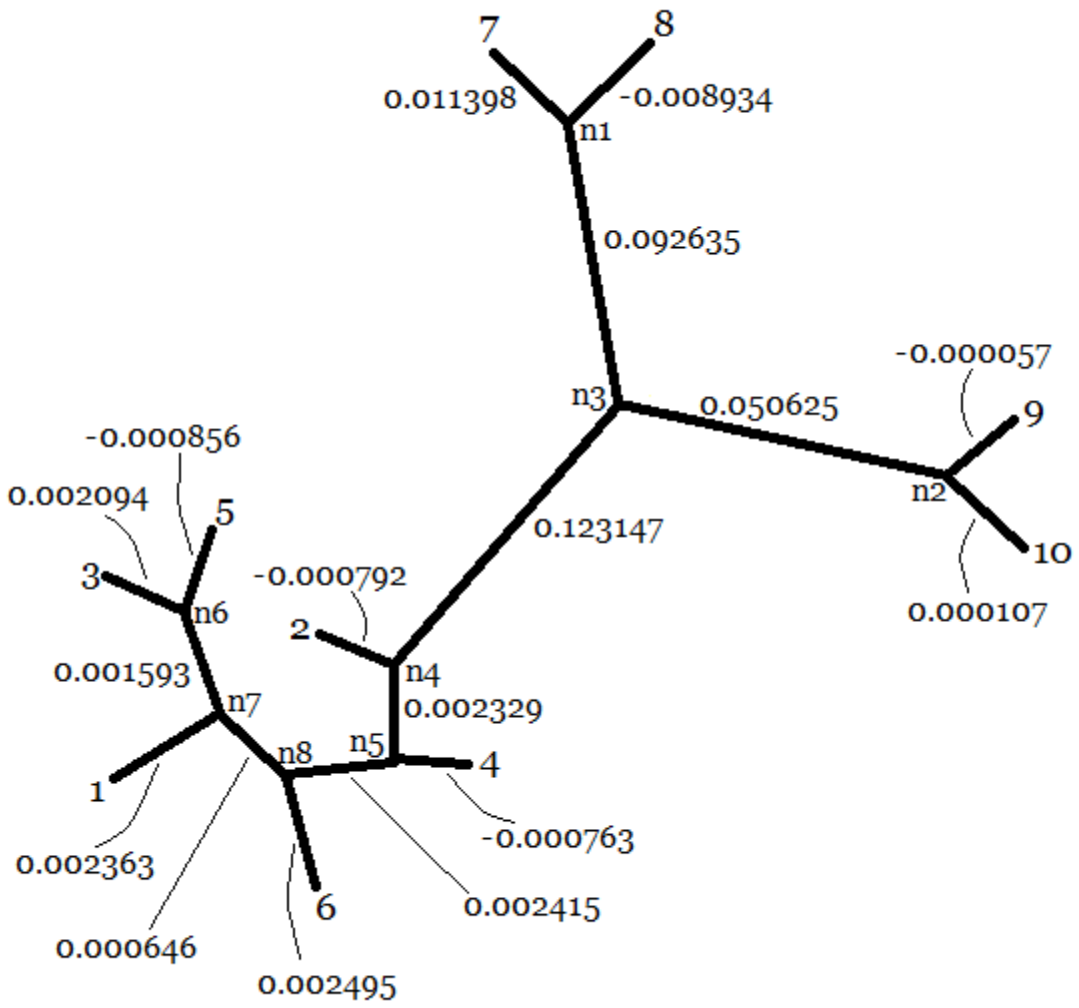

Fig. D.1. Phenogram with labeled nodes. Branch lengths not to scale. See Table D.2 for extended branch lengths.

REFERENCES

Adler, P.H. 2005. Black flies, the Simuliidae. Pp. 127-140. In W. C. Marquardt (ed.). Biology of Disease Vectors, 2nd edition. Elsevier Academic Press, San Diego, CA.

Adler, P.H. 2009. Biting flies of Necedah National Wildlife Refuge, Wisconsin, with reference to whooping cranes. Final Report to Natural Resources Foundation of Wisconsin, Madison, WI. 13 pp.

Adler, P.H. and R.W. Crosskey. 2012. World blackflies (Diptera: Simuliidae): A comprehensive revision of the taxonomic and geographical inventory [2012]. Available at < http://entweb.clemson.edu/biomia/pdfs/blackflyinventory.pdf>. 119 pp.

Adler, P.H., C.L. Brockhouse, and D.C. Currie. 2003. A new species of black fly (Diptera: Simuliidae) from Nova Scotia. Proceedings of the Entomological Society of Washington 105:9-12.

Adler, P.H., D.C. Currie, and D.M. Wood. 2004. The Black Flies (Simuliidae) of North America. Cornell University Press, Ithaca, NY. xv + 941 pp.

Adler, P.H., J. Barzen, E.W. Gray, R. King, and J. Smink. 2010a. Black fly surveys and pilot *Bti* study, with reference to the species that attack whooping cranes in Wisconsin. Final Report to Natural Resources Foundation of Wisconsin, Madison, WI. 35 pp.

Adler, P.H., R.A. Cheke, and R.J. Post. 2010b. Evolution, epidemiology, and population genetics of black flies (Diptera: Simuliidae). Infection, Genetics and Evolution 10:846-865.

Avise, J.C. 2004. Molecular Markers, Natural History, and Evolution. 2nd ed. Sinauer Associates, Inc. Publishers, Sunderland, MA. 684 pp.

Baldwin, W.F., A.S. West, and J. Gomery. 1975. Dispersal pattern of black flies (Diptera: Simuliidae) tagged with ^{32}P. The Canadian Entomologist 107:113-118.

Bauer, F., A. Stübner, C. Neinhuis, and M. Nuss. 2012. Molecular phylogeny, larval case architecture, host-plant associations and classification of European Coleophoridae (Lepidoptera). Zoologica Scripta 41:248-265.

Bedo, D.G. 1977. Cytogenetics and evolution of *Simulium ornatipes* Skuse (Diptera: Simuliidae) I. Sibling speciation. Chromosoma 64:37-65.

Brooks, W., L. Clemency, J. French, B. Hartup, R. Schroeder, D. Staller, and W. Sturgeon. 2010. *Whooping Crane Eastern Partnership Five Year Strategic Plan*. Whooping Crane Eastern Partnership, 13 pp. Available online: <http://www.bringbackthecranes.org/design/pdf/WCEP_5YearStrategicPlan.pdf>.

Brown, D.M., R.A. Brenneman, K.P. Koepfli, J.P. Pollinger, B. Milá, N.J. Georgiadis, E.E. Louis, Jr., G.F. Grether, D.K. Jacobs, and R.K. Wayne. 2007. Extensive population genetic structure in the giraffe. BMC Biology 5**(**57); 13 pp.

Claridge, M.F. 2009. Species are Real Biological Entities, in Contemporary Debates in Philosophy of Biology (eds F.J. Ayala and R. Arp). Wiley-Blackwell, Oxford, UK.

Coluzzi, M., A. Sabatini, A. della Torre, M.A. Di Deco, and V. Petrarca. 2002. A polytene chromosome analysis of the *Anopheles gambiae* species complex. Science 298:1415-1418.

Commission for Environmental Cooperation Working Group. 1997. Ecological regions of North America – toward a common perspective. Commission for Environmental Cooperation, Montreal. 71 pp.

Costa, F.O. and G.R. Carvalho. 2010. New insights into molecular evolution: prospects from the Barcode of Life Initiatie (BOLI). Theory in Biosciences 129:149-157.

Dick, C.W. 2007. High host specificity of obligate ectoparasites. Ecological Entomology 32:446-450.

Ekrem, T., E. Stur, and P.D.N. Hebert. 2010. Females do count: Documenting Chironomidae (Diptera) species diversity using DNA barcoding. Organisms Diversity and Evolution 10:397-408.

Elmer, K.R., J.A. Dávila, and S.C. Lougheed. 2007. Cryptic diversity and deep divergence in an upper Amazonian leaflitter frog, *Eleutherodactylus ockendeni*. BMC Evolutionary Biology 7:247. 14 pp.

Excoffier, L. and H.E. L. Lischer. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564-567.

Faria, R. and A. Navarro. 2010. Chromosomal speciation revisited: rearranging theory with pieces of evidence. Trends in Ecology and Evolution 25:660-669.

Felsenstein, J. 2004. PHYLIP (Phylogeny Inference Package) version 3.6. *Distributed by the author. Department of Genome Sciences, University of Washington, Seattle*.

Forbes, S.A. 1912. On black-flies and buffalo-gnats (*Simulium*) as possible carriers of pellagra in Illinois. Report of the Illinois State Entomologist 27:21-55.

Gregory, T.R. 2011. Animal Genome Size Database. http://www.genomesize.com.

Golini, V.I. and K. Rothfels. 1984. The polytene chromosomes of North American blackflies in the *Eusimulium canonicolum* group (Diptera: Simuliidae). Canadian Journal of Zoology 62:2097-2109.

Hart, M.W. 2011. The species concept as an emergent property of population biology. Evolution 65:613-616.

Jackson, B.C. 2011. Recombination-suppression: how many mechanisms for chromosomal speciation?. Genetica 139:393-402.

Kachvoryan, E.A., L.A. Chubareva, N.A. Petrova, and L.S. Mirumyan. 1996. Frequency changes of B chromosomes in synanthropic species of bloodsucking blackflies (Diptera, Simuliidae). Genetika 32:637-640.

Keesing, F., L.K. Belden, P. Daszak, A. Dobson, C.D. Harvell, R.D. Holt, P. Hudson, A. Jolles, K.E. Jones, C.E. Mitchell, S.S. Myers, T. Bogich, and R.S. Ostfeld. 2010. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature 468:647-652.

Khadem, M., R. Camacho, and C. Nóbrega. 2011. Studies of the species barrier between *Drosophila subobscura* and *D. madeirensis* V: the importance of sex-linked inversion in preserving species identity. Journal of Evolutionary Biology 24:1263-1273.

King, R. S. and P. H. Adler. 2012. Development and evaluation of methods to measure populations of black flies (Diptera: Simuliidae) at nests of the endangered whooping crane (*Grus americana*). Journal of Vector Ecology. In press.

Langella, O. 2011. Populations version 1.2.32. *Distributed by the author. Bioinformatics organization, available at http://www.bioinformatics.org/project/?group_id=84.*

Larsen, M.L., J.M. Scriber, and M.P. Zalucki. 2008. Significance of a new field oviposition record for *Graphium eurypylus* (L.) (Lepidoptera: Papilionidae) on *Michelia champaca* (Magnoliaceae). Australian Journal of Entomology 47:58-63.

Malmqvist, B., D. Strasevicius, O. Hellgren, P.H. Adler, and S. Bensch. 2004. Vertebrate host specificity of wild-caught blackflies revealed by mitochondrial DNA in blood. Proceedings of the Royal Society B (Supplement) 271:S152-S155.

McGaugh, S.E. and M.A.F. Noor. 2012. Genomic impacts of chromosomal inversions in parapatric *Drosophila* species. Philosophical Transactions of the Royal Society B 367:422-429.

Michailova, P., E. Warchałowska-Śliwa, E. Szarek-Gwiazda, and A. Kownacki. 2012. Does biodiversity of macroinvertebrates and genome response of Chironomidae larvae (Diptera) reflect heavy metal pollution in a small pond? Environmental Monitoring and Assessment 184:1-14.

Nei, M., 1987 Molecular Evolutionary Genetics. Columbia University Press, New York, NY, USA.

Noor, M.A.F., K.L. Grams, L.A. Bertucci, and J. Reiland. 2001. Chromosomal inversions and the reproductive isolation of species. PNAS 98:12084-12088.

Osei-Atweneboana, M.Y., M.D. Wilson, R.J. Post, and D.A. Boakye. 2001. Temephos-resistant larvae of *Simulium sanctipauli* associated with a distinctive new chromosome inversion in untreated rivers of south-western Ghana. Medical and Veterinary Entomology 15:113-116.

Paetkau, D., W. Calvert, I. Stirling, and C. Strobeck. 1995. Microsatellite analysis of population structure in Canadian polar bears. Molecular Ecology 4:347-54.

Paetkau, D., L.P. Waits, P.L. Clarkson, L. Craighead, and C. Strobeck. 1997. An empirical evaluation of genetic distance statistics using microsatellite data from bear (Ursidae) populations. Genetics 147:1943-1957.

Pramual, P. and K. Wongpakam. 2011. Cytogenetics of *Simulium siamense* Takaoka and Suzuki, 1984 (Diptera: Simuliidae) in Northeastern Thailand. Aquatic Insects: International Journal of Freshwater Entomology 33:171-184.

Pramual, P., B. Gomontean, V. Buasay, N. Srikhamwiang, P. Suebkar, C. Niamlek, Y. Donsinphoem, and K. Chalat-Chieo. 2009. Population cytogenetics of *Chironomus circumdatus* Kieffer, 1921 (Diptera, Chironomidae) from Thailand. Genetica 135:51-57.

Rice, W.R. 1989. Analyzing tables of statistical tests. Evolution 43:223-225.

Richner, H. 1998. Host-parasite interactions and life-history evolution. Zoology 101:333-344.

Rohland, N., D. Reich, S. Mallick, M. Meyer, R.E. Green, N.J. Georgiadis, A.L. Roca, and M. Hofreiter. 2010. Genomic DNA sequences from mastodon and woolly mammoth reveal deep speciation of forest and savanna elephants. PLoS Biology 8(12): e1000564. Doi:10.1371/journal.pbio.1000564

Rothfels, K. 1956. Black flies: siblings, sex, and species grouping. Journal of Heredity 47:113-122.

Rothfels, K. 1981. Cytotaxonomy: Principles and their application to some northern species-complexes in *Simulium*. Pp. 19-29. *In* M. Laird (ed.), Blackflies: the future for biological methods in integrated control. Academic Press, New York. 399 pp.

Rothfels, K. 1989. Speciation in black flies. Genome 32:500-509.

Rothfels, K. H., and R. W. Dunbar. 1953. The salivary gland chromosomes of the black fly *Simulium vittatum* Zett. Canadian Journal of Zoology 31:226-241.

Rothfels, K. and D. Featherston. 1981. The population structure of *Simulium vittatum* (Zett): the IIIL-1 and IS-7 sibling species. Canadian Journal of Zoology 59:1857-1883.

Rothfels, K., R. Feraday, and A. Kaneps. 1978. A cytological description of sibling species of *Simulium venestum* and *S. verecundum* with standard maps for the subgenus *Simulium* Davies (Diptera). Canadian Journal of Zoology 56:1110-1128.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sanderson, C.L., D.R.C. McLachlan, and U. De Boni. 1982. Altered steroid induced puffing by chromatin bound aluminum in a polytene chromosome of the black fly *Simulium vittatum*. Canadian Journal of Genetics and Cytology 24:27-36.

Schaeffer, S. W., A. Bhutkar, B. F. McAllister, M. Matsuda, L. M. Matzkin, P. M. O'Grady, C. Rohde, V.L.S. Valente, M. Aguadé, W.W. Anderson, et al. 2008. Polytene chromosomal maps of 11 Drosophila species: The order of genomic scaffolds inferred from genetic and physical maps. Genetics 179:1601-1655.

Sharakhova, M.V., C. Antonio-Nkondjio, A. Xia, C. Ndo, P. Awono-Ambene, F. Simard, and I.V. Sharakhov. 2011. Cytogenetic map for *Anopheles nili*: Application for population genetics and comparative physical mapping. Infection, Genetics and Evolution 11:746-754.

Shewell, G.E., and F.J.H. Fredeen. 1958. Two new black flies from Saskatchewan (Diptera: Simuliidae). Canadian Entomologist 90:733-738.

Shields, G.F., and M.J. Kratochvil. 2011. A remnant of an incipient speciation event in the *Simulium arcticum* complex (Diptera: Simuliidae). American Midland Naturalist 166:239-251.

Sites, J.W. Jr. and J.C. Marshall. 2004. Operational criteria for delimiting species. Annual Review of Ecology, Evolution, and Systematics 35:199–229.

Stehn, T. 2001. Whooping Crane Recovery Activities, March-August 2001. Whooping Crane Recovery Report, available at <http://www.bringbackthecranes.org/technicaldatabase/recovery/recv2001.html>.

Thomas, C.A. Jr. 1941. The genetic organization of chromosomes. Annual Review of Genetics 5:237-256.

Urbanek, R.P., S.E. Zimorski, A.M. Fasoli, and E.K. Szyszkoski. 2010. Nest desertion in a reintroduced population of migratory whooping cranes. Proceedings of the North American Crane Workshop 11:133-141.

Waser, P.M. and C. Strobeck. 1998. Genetic signatures of interpopulation dispersal. Trends in Ecology and Evolution 13:43-44.

Wheeler, Q.D. and R. Meier. 2000. Species concepts and phylogenetic theory: a debate. Columbia University Press, New York.

White, B.J., F.H. Collins, and N.J. Besansky. 2011. Evolution of *Anopheles gambiae* in relation to humans and malaria. Annual Review of Ecology, Evolution, and Systematics 42:111-132.

Zacharopoulou, A., A.A. Augustinos, W.A.A. Sayed, A.S. Robinson, and G. Franz. 2011. Mitotic and polytene chromosome analysis of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae). Genetica 139:79-90.

Zhimulev, I.F. and D.E. Koryakov. 2009. Polytene Chromosomes. In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd, Chichester. 10 pp. [DOI: 10.1002/9780470015902.a0001183.pub2]

Zhimulev, I.F., E.S. Belyaeva, T.Y. Vatolina, and S.A. Demakov. 2012. Banding patterns in *Drosophila melanogaster* polytene chromosomes correlate with DNA-binding protein occupancy. BioEssays 34:498-508.